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**LA THÈSE A ÉTÉ
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THE METABOLISM AND TOXICOLOGY OF MANGANESE

by

Anton Michael Scheuhammer

Department of Pathology

Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario
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DEDICATION

For Magdalena and Josef.

ABSTRACT

Since manganese (Mn) is both an essential trace metal and, under conditions of excess exposure, a neurotoxic metal, it is important to reach a clearer understanding of how Mn is absorbed, distributed, accumulated, and excreted, as well as to determine the effects of Mn on various metabolic processes, particularly in the brain.

In the present series of studies, Mn was administered to rats via injection (ip or iv), or orally in drinking water.

Mn administered as a single iv dose was recovered primarily from the bile and liver, which together comprised about 80% of the recovered dose, 3 hrs after administration. Mn did not bind to presynthesized hepatic metallothionein. Although the simultaneous administration of Mn + L-dopa caused a significant decrease in the biliary excretion of Mn, no evidence was found to support the contention that the chelation of Mn by L-dopa is a significant factor influencing the distribution and excretion of either Mn or L-dopa.

In plasma, Mn binds extremely poorly to albumin compared to Cd, Zn, and Fe, but binds much better than Cd or Zn to transferrin. The binding of Mn to plasma proteins is affected by pH, the valence state of Mn (Mn^{2+} vs Mn^{3+}), and species differences (human vs rat).

Mn increased in all tissues except liver due to daily ip injections of 3.0 or 3.5 mg Mn/kg for 30 days. Bone and pancreas revealed the largest increases. In blood, increased Mn levels were almost totally accounted for by increases in the erythrocyte fraction. In the brain, certain regions, such as the corpus striatum and midbrain tegmentum, accumulated higher than average levels of Mn compared to other brain regions. Increased Cu concentrations were detected in

plasma, pancreas, duodenum, testes, bone, lung, and in several brain regions of the Mn-treated rats. Also, Zn levels in plasma and bone were significantly reduced, as were Mg levels in heart and bone. In the corpus striatum, no changes in the concentrations of any neurotransmitter (DA, NE, 5-HT), nor any of their metabolites (DOPAC, HVA, 5-HIAA) were observed, but in the frontal cortex and pons-medulla, DA levels were significantly elevated over control. There was no change in the concentrations of the DA metabolites, however, indicating that the synthesis of DA may be initially affected by subchronic Mn administration but not the rate of degradation. Levels of NE were also elevated in the pons-medulla of Mn-treated rats. D₂ DA-receptor density was unaffected by 30 days of Mn treatment, nor did the addition of high concentrations of Mn²⁺ (up to 10⁻² M) to striatal homogenates in vitro cause any reduction in receptor number. Significant pathological changes characterized by a pancreatitis-like reaction with destruction of acinar cells were observed in pancreatic tissue from Mn-injected rats. Since other peritoneal organs did not exhibit pathological changes, it is suggested that intraperitoneally injected Mn salts are selectively toxic to pancreatic tissue.

Upon cessation of Mn treatment, excess Mn was spontaneously eliminated from all tissues examined. After two weeks post-Mn, kidney levels had virtually returned to normal, and levels in testes and muscle were much reduced. In the brain, Mn levels were reduced, but were still more than 2X normal at the end of the experiment. Within the Mn-exposed group, rats subsequently treated with EDTA excreted 12X as much Mn/day in their urine as those not treated with EDTA. However, EDTA treatment did not result in an enhancement in the natural rate of elimination of

Mn from any soft tissue except skeletal muscle. A highly significant positive correlation was noted between the urinary excretion of Mn and Fe under the influence of EDTA treatment.

When Mn was administered orally (2800 ppm in drinking water), the accumulation of the metal in tissues was very slow. Two to three times more Mn was accumulated due to ip injection for 30 days than to oral exposure for 200 days. Other differences between oral and ip administration include: proportionally much higher levels of Mn in bone and pancreas as a result of ip injection; the absence of pathological changes in pancreatic tissue due to the oral administration of Mn; and significant increases through time in the Mn content of liver due to chronic oral exposure, an effect which was not observed after ip injections. No significant changes in the levels of striatal DA, DOPAC, HVA, NE, 5-HT, or 5-HIAA were observed due to the oral administration of Mn.

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Finally, I would like to thank God that this is finally finished.

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especially in the developing animal in which cartilage formation and, therefore by necessity, mucopolysaccharide synthesis is normally commencing at a very rapid rate.

Neonatal ataxia characterized by incoordination, loss of equilibrium, and head retraction, is associated with Mn deficiency in several animal species (Norris and Caskey, 1939; Hill et al., 1950; Hurley et al., 1958). This condition cannot be reversed by Mn supplementation after birth. The ataxia results not from a CNS defect, as might at first be expected, but from faulty embryonic development of the inner ear, particularly the otoliths of the utricular and saccular maculae (Asling et al., 1960), bony formations which are involved in the proper maintenance of balance and equilibrium. The defective otoliths reveal abnormally low quantities of acid mucopolysaccharides (Shrader and Everson, 1967), thus it appears that the neonatal ataxia of Mn deficiency may be attributable to the same basic biochemical defects which are responsible for the gross skeletal abnormalities discussed above.

Interestingly, a mutant gene affecting coat color in mice - 'pallid' - also produces a congenital ataxia very similar to that observed with Mn-deficient normal mice. Supplementing the diet of the mutants with high levels (1500-2000 ppm) of Mn during pregnancy prevented the occurrence of ataxia in the offspring (Erway et al., 1971). It was later determined (Cotzias et al., 1972) that 'pallid' mice exhibited a slower rate of loss of injected ⁵⁴Mn and a higher relative retention in the liver compared with other tissues, than normal controls. The bones and brains of 'pallid' mice contained less Mn than black controls and the bones were more fragile. It was concluded that

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ABBREVIATIONS

AAS	atomic absorption spectrophotometry
ACh	acetylcholine
AChE	acetylcholinesterase
ATP	adenosine triphosphate
BAL	British Anti-Lewisite (2,3-dimercaptopropanol)
cAMP	cyclic adenosine 3',5'-monophosphate
Ci	curie
CNS	central nervous system
DA	dopamine
DMPS	2,3-dimercapto-1-propanesulfonate
DOPAC	3,4-dihydroxyphenylacetic acid
DTE	dithioerythritol
EDTA	ethylenediaminetetracetic acid
g	gram
GABA	γ -aminobutyric acid
GABA-T	GABA-transaminase
GAD	glutamic acid decarboxylase
GI	gastrointestinal
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine (serotonin)
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
HMW	high molecular weight
HPLC	high performance liquid chromatography
HVA	homovanillic acid
ip	intraperitoneal
iv	intravenous
kg	kilogram
l	litre
L-dopa	L-3,4-dihydroxyphenylalanine
MAO	monoamine oxidase
MEM	minimum essential medium
mg	milligram
MT	metallothionein

MW	molecular weight
nA	nanoampere
NE	norepinephrine
NEM	N-ethylmaleimide
ng	nanogram
ppb	parts per billion
ppm	parts per million
QNB	quinuclidinyl benzilate
SPIRO	spiroperidol (spiperone)
S.D.	standard deviation
S.E.	standard error
TH	tyrosine hydroxylase
TLV	threshold limit value
Tris	tris (hydroxymethyl) aminomethane

CHAPTER ONE

LITERATURE REVIEW

1.1 Manganese in Biological Systems

1.1.1 Manganese in the Ecosystem

The mean concentration of manganese (Mn) in the earth's crust has been estimated to be approximately 1000 ppm, making it the 4th most abundant trace element, after aluminum, iron, and titanium (Goldschmidt, 1958). Because its chemical behavior is similar to that of iron (Fe), Mn is often found in close association with Fe in the environment. Metallic Mn does not occur in nature, but Mn ore deposits in the form of pyrolusite (MnO_2), hausmannite (Mn_3O_4), braunite (a mixture of Mn_2O_3 and MnSiO_3) and rhodochrosite (MnCO_3) are widespread throughout the tropical, subtropical, and warmer temperate zones of the earth. These Mn deposits have provided the material base for a world-wide Mn-mining industry for over 200 years. Currently, the U.S.S.R. is the leading world producer of Mn ores, followed by the Republic of South Africa, Gabon, Brazil, Australia, and India.

The normal range for the Mn content of soils in the U.S. and Canada is between 100 and 4000 ppm (Cannon and Anderson, 1971; Warren et al., 1971; Bowen, 1966), however, much higher concentrations may be found in soil on or near Mn ore deposits. In such locales, Mn may compose 5-15% of the total soil weight, and acidic soils contain higher levels of the metal than neutral or basic soils (Vinogradov, 1959).

The predominate chemical forms of Mn in soil are trivalent and tetravalent oxides which are highly insoluble and generally unavailable for incorporation into the biosphere. Levels of dissolved Mn in the

ground water are low, usually less than 50 ppb, virtually all of which is Mn^{2+} . Most divalent Mn salts are soluble in aqueous media, thus the bioavailability of Mn from soil is dependent upon the reduction of $Mn^{4+}/Mn^{3+} \rightarrow Mn^{2+}$, a process which is favored only in acidic media ($pH < 6$). Plant roots, as well as certain bacteria, are able to reduce MnO_2 to soluble absorbable forms (Vinogradov, 1959; Passioma and Leeper, 1963). In this way, terrestrial plants initiate the bioconcentration of Mn.

The average Mn content of freshwater rivers and lakes has been determined to be between 1 and 200 ppb (Durum and Haffty, 1963; Kleinkopf, 1960; Kononov et al., 1966). These levels are several-fold greater than those reported for seawater, values for which typically fall between 0.4 and 5.0 ppb (Goldberg, 1965; Goldberg and Arrhenius, 1958; Slowey, 1966). Marine sediments, however, contain ~ 10,000 ppm Mn (Green, 1959) and manganese oxide "nodules" which contain 20-80% Mn can be found, often in high concentrations, over large areas of the Pacific, Atlantic, and Indian Ocean floors beyond the continental shelves (Mero, 1965; Goldberg, 1965). Thus, it appears that rain water leaches soluble Mn from the land, and rivers carry it to the sea where it is oxidized and precipitated on the ocean floor. Several genera of bacteria common to oceanic muds are able to precipitate manganese oxides from manganese salts (Hewett, 1932).

Marine phyto- and zoo-plankton, and marine plants, concentrate Mn from seawater 100-1000-fold and may contain up to 400 ppm Mn on a wet weight basis. Thus, marine animals which feed on plankton and vegetation may be exposed to high levels of Mn over the course of their lives. Vinogradov (1953) was one of the first to suggest that the

evolutionary development of the hepatopancreas in mollusks may have been a response to the need for a homeostatic mechanism which could regulate the accumulation of Mn, a line of thought that was later pursued by Gotzias and Papavasiliou (1964).

1.1.2 Sources of Exposure

There are essentially three routes of human exposure to Mn from the environment: air, water, and food.

Except under certain occupational situations (which will be further discussed in Section 1.3.2), levels of Mn in the ambient air are very low, generally below $0.1 \mu\text{g}/\text{m}^3$ in non-urban air (EPA, 1971). With the rare exception of a volcanic explosion, high levels of Mn in the air depend solely on manmade sources of emission. These sources include ferromanganese alloy production plants, steel foundries, ore crushing facilities, dry-cell battery manufacturing plants, and plants for the production of Mn-containing chemicals such as permanganates and certain fertilizers. Emissions from these industrial sources can occasionally cause local pollution problems where they are not adequately controlled. A classic example of manganese pollution of the atmosphere occurred at Sauda, Norway, where air in the vicinity of a ferroalloy plant periodically contained as high as $60 \mu\text{g Mn}_3\text{O}_4/\text{m}^3$ (Bockmann, 1939).

Within the last 10 years, concern has been expressed regarding the use of Mn-tricarbonyl compounds. (methylcyclopentadienyl manganese tricarbonyl; MMT) as fuel additives to improve combustion. In the U.S., regulatory actions have prevented the use of MMT in gasoline, but not as a fuel additive in general (Costle, 1978), and in Canada, MMT is still used as a lead substitute in gasoline. The increased use of MMT inevitably leads to increased environmental levels of Mn, mainly as particulate Mn_3O_4 (Ter Haar et al., 1975). Mena (1974) has estimated that 0.5 g Mn/gallon of gasoline would increase the current inhalation of Mn from 2 $\mu\text{g/day/person}$ to 6-24 $\mu\text{g/day}$. It is doubtful that these levels would pose any significant threat to the health of the general population, however it is possible that sensitive sub-populations may be at risk. In this regard, it is known that the retention of Mn (Cotzias et al., 1974), the uptake of Mn by the brain (Mena, 1974), and the neurotoxic effects of Mn (Chandra and Shukla, 1978) are all potentiated in neonatal and growing mammals as compared with adults.

The levels of Mn found in drinking water are, like normal ambient air values, very low (0.02-1.5 ppm in U.S. rivers; Kopp and Kroner, 1969). Therefore, unless contamination of the drinking water supply has occurred, as has been occasionally reported (Kawamura et al., 1941), the daily consumption of Mn from drinking water is negligible.

The major source of Mn for the general population is from food. The average daily intake for adults is ~ 5 mg. (Wenlock et al., 1978), whereas that of infants during the first six months is considerably lower (2.5-75 $\mu\text{g/day/kg}$ body wt.) due to the low concentration of Mn in

milk (McLeod and Robinson, 1972b). Cereal grains contain the highest Mn concentrations of the common foodstuffs (1-35 mg/kg) whereas meat products contain relatively lower amounts (0.3-1.5 mg/kg) (Wenlock et al., 1979). Tea leaves contain relatively high levels of Mn (350-900 mg/kg).

1.1.3 Absorption

The major route of absorption of Mn is the gastrointestinal tract. Early studies by Greenberg et al. (1943) with radiomanganese indicated that only about 4% of an orally administered dose was absorbed by rats. A study by McLeod and Robinson (1972a) on four young women over a 27 day period revealed that daily dietary intakes of Mn were between 2 and 4 mg with retentions between 0.05 and 0.46 mg, indicating an average absorption of about 8%.

Although the biochemical mechanisms governing the intestinal absorption of Mn are poorly understood, the antagonistic relationship between Mn and Fe is well established. Over 40 years ago, it was already recognized that the addition of ferric citrate to the diet accentuated the severity of perosis caused by Mn-deficiency in chickens (Wilgus and Patton, 1939). Later, the reciprocal observation was made by Hartman et al. (1955) and Matrone et al. (1959). In these studies, the addition of extra Mn to normal diets retarded the rate of hemoglobin regeneration in rabbits, pigs and lambs which had previously been made anemic through Fe deficiency. This effect could be overcome by the simultaneous addition of extra Fe to the diets. Diez-Ewald et al.

(1968), using an isolated intestinal loop technique, found that the intestinal absorption of Fe was approximately 300% greater in rats which had been fed a diet high in Mn (33 mg MnCl_2/g) for 5 weeks as compared to controls receiving a standard diet (50.5 $\mu\text{g Mn/g}$). Concurrently, the hepatic Fe concentration was reduced to about 30% of control. When the absorption of ^{54}Mn was examined, it was observed that Fe-loaded animals absorbed less and Fe-deficient animals absorbed more ^{54}Mn than normal controls. Thus, when Fe absorption was increased due to Fe deficiency, so was Mn absorption increased, and decreased Fe absorption in Fe-loaded animals was associated with decreased Mn absorption. In another study utilizing perfused open-ended intestinal loops, Thomson et al. (1971) found that in Fe-loaded rats, Mn was absorbed largely by diffusion throughout the length of the small intestine, whereas in Fe-deficiency, Mn absorption was increased in the duodenum and jejunum due to the enhanced activity of a transport system which could be competitively inhibited by Fe. Mena et al. (1969) showed that, in humans, anemia was associated with a high intestinal absorption of both ^{59}Fe and ^{54}Mn , and a high level of ^{55}Mn in erythrocytes as compared to normal controls.

Mn introduced into the intestine by biliary secretion is known to enter the enterohepatic circulation (Klassen, 1974). This bile-bound Mn is more easily absorbed from the gut than MnCl_2 (Cikrt, 1973). The chemical form of Mn in the bile is not known, but it is probably bound to low molecular weight substances (Cikrt and Tichy, 1972). The biliary excretion of Mn is discussed further in Section 1.1.5.

There are very few reports which have examined the absorption of Mn from the lung even though inhalation is the major route of exposure to Mn under occupational conditions. Mena et al. (1969) reported that,

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in human volunteers inhaling a nebulized solution of $^{54}\text{Mn}_2\text{Cl}_2$ or suspension of $^{54}\text{Mn}_2\text{O}_3$, the radioactivity located in the lungs showed a fairly rapid decline during the first day, with a concurrent increase in the radioactivity recorded over the epigastrium. Later, radioactivity shifted to the right hypochondrium and, eventually, to the hypogastrium. The fate of the oxide was identical to that of the chloride. Collection of feces for four days resulted in the recovery of 40-70% of the radioactivity initially located in the lungs. These data were interpreted as indicating that Mn in the lung is rapidly deglutated by ciliary action and transferred to the gastrointestinal tract with only a minor portion of the administered dose actually absorbed by the lung. However, another possible explanation is that significant absorption of Mn by the lung does occur followed by the rapid translocation of Mn to the liver and its subsequent appearance in the GI tract due to efficient biliary excretion.

The deposition and clearance of particulates in the respiratory tract depends to a large extent on particle size, therefore it is probable that, under actual occupational exposure conditions where the variation in size of Mn-containing dust particles might be considerable, a significant proportion of particles would be cleared rapidly by mucociliary activity whereas smaller particles would be deposited in the lungs resulting in a slower clearance rate of Mn. In accordance with this explanation, Morrow et al. (1966) found a biexponential clearance curve for inhaled $^{54}\text{MnO}_2$ in normal human subjects with a rapid-phase half-life of 2.8 hours and a slow-phase half-life of 66 days. In dogs, the corresponding values were < 1 day and 34 days respectively (Morrow et al., 1964).

Absorption of Mn through the skin does not occur to a significant extent (WHO, 1981).

1.1.4 Tissue and Subcellular Distribution

An extensive survey by Schroeder et al. (1966) revealed little variation in the Mn content of human tissues due to geographic origin or to age. Tissue specimens from Japan, India, Africa, Switzerland, and from across the U.S.A. had similar concentrations of Mn, and there was little or no trend for decrease or accumulation with aging. There were, however, indications that the Mn concentrations of organs were considerably higher than average in utero and shortly after birth than in the adult.

It has been estimated that a normal 70 kg man has a total body burden of Mn of 12-20 mg (Cotzias, 1958). Organs with the highest concentrations are the liver, pancreas, and kidney, in descending order ($2.0 \pm 0.9 \mu\text{g/g}$ wet wt.), whereas skeletal muscle has a very low Mn content ($<0.1 \mu\text{g/g}$ wet wt.) (Tipton and Cook, 1963). Data from a variety of animal species reveal a similar distribution pattern to that found in man (Schroeder et al., 1966; Fore and Morton, 1952).

In blood, the concentration of Mn in erythrocytes is several-fold greater than that of serum or plasma ($15-25 \mu\text{g/l}$ vs. $0.5-1.5 \mu\text{g/l}$ respectively) (Papavasiliou and Cotzias, 1961). In red cells, it is associated with the hemolysate in a non-dialysable, non-exchangeable form which is not available for chelation with EDTA; it is probably

present as an Mn-porphyrin (Borg and Cotzias, 1958). There is disagreement in the published literature concerning the nature of Mn-binding ligands in serum and plasma. Some researchers feel that Mn binds primarily to serum albumin (Nandadkar et al., 1973), others believe transferrin to be the major binding ligand (Keefer et al., 1970), and still others have proposed the existence of a specific β_1 -globulin which serves as the Mn-transport protein (transmanganin) in serum (Cotzias and Bertinchamps, 1960). Further investigations are required to resolve these discrepancies.

In normal human subjects, intravenously injected carrier-free $^{54}\text{Mn}^{2+}$ is cleared very rapidly from the blood with a half-life of 1-2 minutes and it is eliminated from the body with a half-life of about 40 days, although in healthy Mn miners exposed to Mn daily, the turnover is more rapid with a half-life of about 15 days (Cotzias et al., 1968). This finding indicates that the turnover of Mn is dependent upon the level of exposure and body burden of the metal, an observation which was confirmed in rats (Britton and Cotzias, 1966) for which the turnover rate of parenterally administered ^{54}Mn increased as the level of stable Mn (^{55}Mn) in the diet was increased.

Animal studies have also provided information as to the fate of ^{54}Mn through time within the body after intravenous injection. Using rhesus monkeys injected with carrier-free ^{54}Mn , Dastur et al. (1971) determined that Mn initially distributed to the highly cellular organs such as liver, pancreas and kidney but that, with the passage of time, the metal was discharged from these organs (and from the body as a whole) resulting in increasingly higher proportions of the remaining radioactivity becoming associated with the CNS and endocrine glands.

Six hours after injection, the distribution of radioactivity/g tissue was as follows: liver > kidneys > pancreas > adrenals > thyroid > brain. By 30 days, this pattern had changed to become: pancreas > adrenals > thyroid > liver > kidneys > brain, and by 278 days, the distribution of remaining activity was adrenals > thyroid > pancreas > brain > kidneys > liver. It was concluded that, while the whole body and most tissues appeared to discharge Mn with the passage of time, first rapidly and then more slowly, the CNS showed persistent levels of radioactivity indicating an extremely long half-life for Mn in nervous tissue, and perhaps an inability of neurons to eliminate excess Mn. This is not necessarily true, however, since turnover may increase dramatically in tissues as exposure level increases (Britton and Cotzias, 1968).

In the brain, heavy metals reveal regional patterns of scarcity and abundance (Haug, 1973). Mn is no exception. In the human brain, normal Mn concentrations vary between 0.5 and 3.0 $\mu\text{g/g}$ wet weight with the highest levels in the caudate-putamen and cerebellar cortex and the lowest in white-matter regions such as the corpus callosum, pons, and cerebellar white (Iwata et al., 1976; Smeyers-Verbeke et al., 1976). In rat brain, the highest normal levels of Mn were reported to be associated with the hypothalamus, olfactory bulbs, and midbrain (Donaldson et al., 1973). There are no data describing the regional accumulation of Mn in either human or animal brain after chronic exposure to the metal.

The entry of Mn into the brain is dependent upon the exposure level, iron status, and age. Animals exposed to high levels of Mn during the preweanling period show a much higher accumulation of the

metal in CNS tissue than do adults similarly exposed, probably because of immature blood-brain barrier mechanisms (Rehnbarg et al., 1981).

Similarly, studies in anemic rats have demonstrated that the entry of injected ^{54}Mn into the brain is increased by 60-160% as compared to controls (Mena, 1974).

As is the case for other heavy metals, Mn is generally found in higher than average concentrations in pigmented tissues such as the retina, conjunctiva, and dark hair and skin (Tauber and Krause, 1943; Cotzias et al., 1964). Black human hair can contain as high as 40 $\mu\text{g/g}$ Mn (Cotzias et al., 1964). In pigmented tissues, Mn (and other metals) are associated with melanosomes and pigment granules (Kikkawa and Fujito, 1955; Seiji et al., 1963; Cotzias et al., 1964).

Subcellularly, Mn tends to be associated with the mitochondrial and nuclear fractions and relatively low concentrations are found in the cytosol (Maynard and Cotzias, 1955; Edwards et al., 1961).

1.1.5 Excretion

The homeostatic control of manganese in the body is very efficient and takes place primarily via variable excretion by the liver into the bile. In rats, intravenously injected ^{54}Mn is cleared very rapidly from the blood and radioactivity consequently increases sharply in the bile (Bertinchamps et al., 1966). Within 48 hrs of injection, over 75% of a large dose (10 mg/kg) of Mn will have been eliminated from the body via the feces (Klaassen, 1974b). Urinary excretion is negligible even in

Mn-loaded animals (Papavasiliou et al., 1966). The swift passage of Mn from blood to liver to bile is in contrast to the relatively slow rates of biliary excretion of other, essential metals such as zinc (Barrowman et al., 1973), copper (Neumann et al., 1962), and iron (Dubach et al., 1955), as well as non-essential metals such as arsenic (Klaassen, 1974a), lead (Blaxter and Cowie, 1946; Klaassen and Shoeman, 1974), mercury (Klaassen, 1976), and cadmium (Cherian and Vostal, 1977; Doyle et al., 1974; Klaassen and Kotsonis, 1977). Although the molecular mechanisms by which the liver clears Mn from the blood and secretes it into the bile are unknown, the process is swift and is accomplished against a concentration gradient, implicating the existence of some form of active transport system (Klaassen, 1974b). In addition, the excretory pathway is extremely specific for Mn. Other metals are unable to antagonize the excretion of Mn, whereas administration of stable Mn (^{55}Mn) is able to increase the biliary excretion of ^{54}Mn (Cotzias and Greenbough, 1958).

Biliary ligation diminished but did not abolish the total-body loss of ^{54}Mn , whereas rectal ligation completely abolished the excretion of ^{54}Mn (Papavasiliou et al., 1966). This finding indicates that, although the liver may be the major organ responsible for the excretion of Mn, extra-hepatic routes of excretion also exist. Auxiliary excretion via the gut (pancreas and small intestine) is especially evident upon saturation of the hepatic system (Bertinchamps et al., 1966),

The liver can remove Mn more efficiently after intraportal administration, than after systemic administration. This "first pass" effect is evident in rats, for example, in which the systemic

availability of Mn following portal administration is 30-35% less than after systemic administration (femoral vein) of low doses of Mn, and a higher dose of Mn is required to produce death when given portally than when administered systemically (Thompson and Klaassen, 1982). The greater ability of the liver to extract Mn from the portal blood may thus have toxicological significance in that it may protect against Mn toxicity after oral intake of the metal. On the other hand, Mn absorbed by inhalation may be retained in the tissues of the body to a greater extent since this route of exposure bypasses the portal circulation.

The administration of high doses of Mn, especially in conjunction with bilirubin, can cause cholestasis (Witzleben et al., 1968; Witzleben, 1972; Klaassen, 1974b). Since bilirubin has been shown to form chelates with metals (Kuenzle et al., 1972), it is thought that Mn-induced decreases in bile flow may be due to a precipitation of bile salts leading to a resultant clogging of the bile canaliculi. It is not known how the liver handles Mn after the induction of cholestasis.

1.2 Metabolism and Biochemistry of Mn

1.2.1 Mn and Enzyme Activity

Many enzymes which require a divalent metal cation for their activity can be activated by Mn^{2+} . Generally, this activation is non-specific, although certain enzymes may require Mn^{2+} for optimal activity, for example, adenylate cyclase in rat brain (Walton and

Baldessarini, 1976). Those enzymes which can be non-specifically activated by Mn^{2+} include a variety of hydrolases, kinases, decarboxylases, and transferases (Vallee and Coleman, 1964).

In contrast to those enzymes which may be activated by Mn^{2+} , the number of Mn-metalloenzymes, that is, enzymes in which Mn is a tightly bound structural constituent, are relatively few. The most extensively studied Mn metalloenzyme is pyruvate carboxylase, a mitochondrial enzyme which catalyzes the ATP-dependent addition of CO_2 to pyruvate. It contains 4 moles of tightly bound Mn per mole of protein (MW \sim 500,000) (Scrutton et al., 1972).

Arginase hydrolyzes arginine to urea and ornithine. It binds 4 moles of Mn per mole of enzyme, with Mn bound tightly at two of the binding sites. Mn cannot be removed from these sites without denaturing the enzyme (Hirsch-Kolb, 1971).

The catalytic activity and stabilization of the quaternary structure of glutamine synthetase require Mn^{2+} or Mg^{2+} , although the affinity of the enzyme for Mn^{2+} is 400 times that for Mg^{2+} (Denton and Ginsburg, 1969). Glutamine synthetase binds 4 moles of Mn per mole of protein and probably exists as an Mn enzyme in vivo (Wedler, 1982).

Avimanginin, isolated from avian liver by Scrutton (1971), contains 1 mole of bound Mn(III) per mole protein. The enzyme's function is not precisely known, but it is probably a superoxide dismutase (Utter, 1976). Some other superoxide dismutases are known to contain tightly bound Mn(III), along with Fe, Cu and/or Zn (Keele et al., 1970).

1.2.2 Effects of Mn Deficiency

Mn is an essential trace element. Over the past 50 years, the effects of Mn deficiency have been studied in several animal species. A few effects are common in all species. These include: 1) skeletal abnormalities (perosis), 2) postural defects (ataxia), and 3) reproductive abnormalities.

The skeletal abnormalities caused by Mn deficiency are particularly prominent during the development of the long bones in neonates. The condition was first described in the chick (Wilgus et al., 1936), and later in rats (Amdur et al., 1945), guinea pigs (Everson et al., 1959), swine (Plumlee et al., 1956), and cattle (Rojads et al., 1965). It is characterized by a severe shortening and thickening of the long bones, and a marked dysplasia of the epiphyseal plate (Hurley and Asling, 1963). Impairment of the calcification process per se is not a primary causal factor in the bone abnormalities observed in Mn deficiency. It is the chondrogenic process rather than the osteogenic process in which Mn participates, and it is through a drastic reduction in the mucopolysaccharide content of cartilage during Mn deficiency that the skeletal abnormalities are mediated (Leach and Muenster, 1962; Tsai and Everson, 1967). The impairment in mucopolysaccharide synthesis associated with Mn deficiency has been related to the activation of glycosyl transferases. These are enzymes involved in the transfer of sugars from sugar nucleotides to a variety of acceptors and they are important in the synthesis of polysaccharides and glycoproteins. They require a metal ion for optimum activity, and Mn is usually the most effective ion (Leach, 1971). Thus, under conditions of Mn deficiency, chondroitin sulphate synthesis might be expected to be severely impaired

especially in the developing animal in which cartilage formation and, therefore by necessity, mucopolysaccharide synthesis is normally commencing at a very rapid rate.

Neonatal ataxia characterized by incoordination, loss of equilibrium, and head retraction, is associated with Mn deficiency in several animal species (Norris and Caskey, 1939; Hill et al., 1950; Hurley et al., 1958). This condition cannot be reversed by Mn supplementation after birth. The ataxia results not from a CNS defect, as might at first be expected, but from faulty embryonic development of the inner ear, particularly the otoliths of the utricular and saccular maculae (Asling et al., 1960), bony formations which are involved in the proper maintenance of balance and equilibrium. The defective otoliths reveal abnormally low quantities of acid mucopolysaccharides (Shrader and Everson, 1967), thus it appears that the neonatal ataxia of Mn deficiency may be attributable to the same basic biochemical defects which are responsible for the gross skeletal abnormalities discussed above.

Interestingly, a mutant gene affecting coat color in mice - 'pallid' - also produces a congenital ataxia very similar to that observed with Mn-deficient normal mice. Supplementing the diet of the mutants with high levels (1500-2000 ppm) of Mn during pregnancy prevented the occurrence of ataxia in the offspring (Erway et al., 1971). It was later determined (Cotzias et al., 1972) that 'pallid' mice exhibited a slower rate of loss of injected ⁵⁴Mn and a higher relative retention in the liver compared with other tissues, than normal controls. The bones and brains of 'pallid' mice contained less Mn than black controls and the bones were more fragile. It was concluded that

the 'pallid' gene expresses itself as an impairment in the transport of Mn through the body resulting in Mn-deficient tissues despite ample concentrations of Mn in the liver. The transportation of L-dopa and L-tryptophan was also impaired in 'pallid' mice implicating a possible link between the metabolism of Mn and the biogenic amines.

Reproductive abnormalities associated with Mn deficiency have been noted in both males and females of several animal species. In female rats, guinea pigs, swine; and cattle, the most prominent characteristic is an irregular or absent estrous cycle and a low survival rate of offspring (Boyer et al., 1942; Everson et al., 1959; Plumlee et al., 1956; Bentley and Philips, 1951). In the male, Mn deficiency is associated with seminal tubular degeneration and the absence of spermatozoa which results in sterility and lack of libido. The precise biochemical lesion(s) responsible for these abnormalities of reproduction is/are unknown.

Except for one possible incident, Mn deficiency in man has not been reported. Doisy (1972) described the case of a man given a synthetic diet from which Mn had been omitted by mistake. After 16 weeks, the patient presented with dermatitis, pigment changes of hair, retarded hair growth, and hypocholesterolemia. The observation of hypocholesterolemia is interesting in light of the probable involvement of Mn in lipid metabolism (see Section 1.2.3).

At a cellular and organelle level, abnormalities caused by Mn deficiency have been observed. Isolated liver mitochondria from Mn-deficient rats or mice were found to take up oxygen at a slower rate than mitochondria from controls, and electron microscopy of these mitochondria revealed ultrastructural changes including elongation and

re-orientation of cristae (Hurley et al., 1970). These original findings were later extended to the cells of other tissues (Bell and Hurley, 1973). In the liver, pancreas, kidney and heart, cell membranes of Mn-deficient mice revealed alterations in integrity. The endoplasmic reticula were swollen, and irregular mitochondria were noted in liver, heart, and kidney. There was an increased level of lipid in liver parenchymal and kidney tubule cells, again implicating the involvement of Mn with lipid metabolism.

1.2.3 Mn and Lipid Metabolism

In vivo, Mn deficiency in animals results in increased fat deposition which can be reduced by subsequent Mn supplementation (Plumlee et al., 1956; Amdur et al., 1946). Choline supplements produce similar results and, furthermore, the changes in liver ultrastructure that arise from Mn deficiency (Bell and Hurley, 1973) are similar to those produced by choline deficiency (Bruni and Hegsted, 1970). These observations have led to the suggestion that choline and Mn are metabolically linked and function in a common pathway responsible for the proper maintenance of cellular membranes.

In vitro, Mn activates (while vanadium inhibits) fatty acid synthesis, and markedly increases cholesterol synthesis in rat liver (Curran, 1954). In the synthetic pathway of cholesterol from acetate, Mn^{2+} is a requirement in at least two enzymatic conversions (Amdur et al., 1957; Tchen, 1957), thus Mn deficiency could conceivably limit the

formation of important precursors of cholesterol. The observation by Doisy (1972) of hypocholesterolemia in an Mn deficient man supports this contention. It should be mentioned also that cholesterol is the precursor of the steroid hormones, thus if Mn deficiency does lead to impaired cholesterol metabolism, then the synthesis of the sex hormones and corticoids might also be impaired. In light of the effects of Mn deficiency on sexual behavior and reproduction (see Section 1.2.2), further investigations designed to elucidate the relationship between Mn and lipid and sterol metabolism are warranted.

1.3 Toxicology of Mn

1.3.1 Acute Toxicity

Since the chief health hazards of Mn result from chronic exposures, relatively few investigations have been undertaken relating to its acute toxicity. Nevertheless, it is known that Mn is one of the least toxic metals when administered as an acute injection. The LD_{50} of $MnSO_4 \cdot 4H_2O$ in mice is 500-600 mg Mn/kg ip, whereas for some other metals, the LD_{50} 's are: $ZnSO_4 \cdot 6H_2O$, 40-50 mg/kg iv; $CuSO_4 \cdot 5H_2O$, 30 mg/kg ip; $Hg \cdot (NO_3)_2$, 8 mg/kg ip (Stokinger, 1981). In accord with the LD_{50} data, Mn^{2+} was found to be less toxic than other divalent cations (Cd^{2+} , Cr^{2+} , Ni^{2+} , V^{2+}) to macrophages in culture (Waters et al., 1975).

In monkeys, the acute administration of Mn as an aerosol resulted in behavioral abnormalities including nervousness, tremor, flexion and extension of the upper limbs, and increased yawning. These symptoms had disappeared 3 weeks after exposure, but subsequently reappeared 5 months later in a more severe form with gross tremors, uncertain gait, and paresis (van Bogaert and Dallemagne, 1945), signs which are fairly characteristic of chronic Mn intoxication in primates (see Section 1.3.2.3).

1.3.2 Chronic Mn Intoxication

Chronic exposure to high levels of Mn is primarily an occupational health hazard. The route of exposure under such conditions is chiefly by inhalation of Mn-containing dusts or fumes. The toxic effects of exposure are twofold: 1) Mn-induced pneumonia, and 2) a debilitating neurological syndrome of extrapyramidal motor dysfunction (manganism).

1.3.2.1 Mn-Induced Pneumonia

Exposure to Mn-containing dusts can produce pulmonary disease in the form of a pneumonia or pneumonitis which is very resistant to antibiotics. The condition appears to be partially caused by both the direct irritative effects of Mn, and increased susceptibility to bacterial invasion of the lung (Lloyd-Davies, 1946).

The incidence of pneumonia in men exposed to Mn while working in a potassium permanganate manufacturing plant was some 30x higher than that of a similar but unexposed group (Lloyd-Davies, 1946). The Mn was mostly in the form of Mn oxides with an air concentration of $0.7-38.3 \mu\text{g}/\text{m}^3$. Particle sizes were under $1 \mu\text{m}$ diameter.

Elstad (1939) reported on the incidence of lobar pneumonia in a Norwegian town in which a ferromanganese plant had been constructed. Before the establishment of the plant, pneumonia was not prevalent, but after operations commenced, the mortality from pneumonia rose to a level 8x as high as that in the rest of the country. The disease struck not only workers in the plant, but also the general populace of the town. The pneumonia was judged to be highly communicable since multiple cases frequently occurred in some homes. Mn concentrations in the town air were generally below $0.05 \mu\text{g}/\text{m}^3$, in the form of Mn_3O_4 , and the particle size was $< 5 \mu\text{m}$.

Green (1970) has suggested that when inhaled particles, including bacteria, are less than $3 \mu\text{m}$ diameter, the phagocytic response by alveolar macrophages constitutes the major pulmonary defense mechanism, and Adkins et al. (1980) noted reduced numbers and viability of alveolar macrophages in mice exposed to Mn_3O_4 (Mn dose, $0.9 \mu\text{g}/\text{m}^3$; particle size $1-3 \mu\text{m}$) by inhalation for 2 hrs. These findings provide support for the contention that exposure to Mn dusts, especially of small particle size, leads to a suppression of those pulmonary defense mechanisms normally mediated by alveolar macrophages, thus making the lung tissue increasingly susceptible to invasion by infectious agents.

1.3.2.2 Human "Manganism"

Couper (1837) was the first to document the neurological movement disorders which have since become typical descriptions of chronic manganese intoxication. On the basis of disorders displayed by five workers at a pyrolusite mill, he expressed the opinion that Mn exposure was able to provoke a condition similar to paralysis agitans (Parkinson's disease). His report, however, fell into obscurity and over 50 years elapsed before descriptions of the syndrome again began to appear in the literature. Most of these reports were by German neurologists (Embsen, 1901; von Jaksch, 1907; Seelert, 1913; Hilpert, 1933; Parnitzke and Peiffer, 1954) who had noted the disorder in brownstone millers. The condition has since been described in miners (Rodier, 1955; Penalver, 1957; Mena et al., 1967), ferromanganese alloy plant workers (Smyth et al., 1973; Saric et al., 1977), dry-battery industry workers (Emara et al., 1971), steel foundry workers (Rosenstock et al., 1971), Mn ore-crushing plant workers (Cook et al., 1974; Greenhouse, 1971) and arc welders (Chandra et al., 1981).

The first symptoms of Mn intoxication are vague but can appear suddenly and progress rapidly. They generally begin to appear after one or two years of exposure, but may develop after one or two months. Individuals who have gone symptomless for 10 years or more rarely develop the syndrome thereafter (Rodier, 1955). Typical early complaints are fatigue, somnolence, apathy, muscle pains, and weakness. In miners, a psychiatric phase characterized by psychomotor disturbances (Mn psychosis) including nervousness and irritability, compulsive singing, dancing, or running, and emotional instability including inappropriate crying or laughing, and hallucinations is common (Rodier,

1955; Mena et al., 1967). Such severe psychiatric manifestations, however, have been noticeably absent from the clinical picture of industrial Mn intoxication in the U.S. where the chief sources of exposure are ore crushing facilities (Greenhouse 1971; Cook et al., 1974). On the other hand, Emara et al. (1971) reported that, in dry-battery industry workers, psychiatric manifestations were the major clinical finding and may be prevalent for years without the subsequent appearance of neurological movement disorders. It has generally been reported, however, that Mn psychosis, if present, soon gives way to a second series of signs and symptoms indicative of selective dysfunction of the extrapyramidal motor system. The major early deficits are postural instability, slowness and clumsiness when moving, and slow, slurred speech. As the syndrome progresses, the bradykinesia increases in severity and is accompanied by retropulsion, propulsion, "cock-walk" or "hen's-gait" in which the patient walks by taking small steps while elevating the heel of the foot and rotating it outward, dystonia of some muscle groups, a masked expressionless face, rigidity, and a rapid intention tremor. These signs, once initiated, usually progress inexorably even after removal from the source of exposure. Although debilitating, Mn poisoning is not fatal. The typical clinical picture of extrapyramidal dysfunction, with the absence of cerebellar and pyramidal tract signs, has resulted in the classification of chronic Mn intoxication as a form of secondary Parkinsonism.

The exposure levels to Mn under occupational conditions have not always been reported, thus it is difficult to correlate the incidence of manganism with the levels of exposure. In Chilean mines, the air concentrations typically ranged from 1-43 mg/m³ during drilling

(Schuler et al., 1957). The incidence of Mn poisoning under these circumstances was 4-25% of the exposed population. In a Mexican mine, the incidence of intoxication was 20% during the first two years after dry drilling of the ore was initiated (Wynter, 1962), but later, with the advent of wet drilling techniques and other safety precautions, the Mn concentration was reduced to $<0.5 \text{ mg/m}^3$, and no further cases of Mn poisoning were detected. Saric et al. (1977) reported the presence of some neurological impairment, particularly of a "subclinical" nature consisting of tremor in the hands, abnormal reflexes, and recurring cramps in the arms and legs, in 17% of workers at a ferroalloy plant where air concentrations of Mn were $0.3\text{--}20 \text{ mg/m}^3$, and in 5.8% of workers in an electrode plant where levels ranged from $2\text{--}30 \text{ }\mu\text{g/m}^3$. Workers in an aluminum rolling mill, where Mn levels were $<0.1 \text{ }\mu\text{g/m}^3$, did not have any signs or symptoms indicative of Mn intoxication. Tanaka and Lieben (1969) surveyed a variety of industries in the U.S. in which a significant exposure to Mn might be present. They found 12 plants out of 75 in which exposure levels exceeded the TLV of 5 mg Mn/m^3 . Of a total of 117 workers from these plants with excessive exposure, 7 were found to have definite signs and symptoms of Mn poisoning.

The question of individual susceptibility must be raised because, even under conditions of extremely high levels of exposure, such as reported by Rodier (1955) of up to 800 mg/m^3 , only a portion of the exposed population ever presents with the signs and symptoms of intoxication. Many workers show no impairment even after 20 years of almost daily exposure. It is thought that the factors which would increase sensitivity are those which would lead to a greater absorption and/or a decreased ability to excrete Mn. Such factors include Fe

deficiency and liver dysfunction due to a variety of causes including alcoholism. However, no definitive correlations have ever been documented.

Rodier (1955) has speculated on the relative toxicity of Mn ores. It was his judgement that the most neurotoxic ores were those containing Mn in its higher valence states, i.e. Mn^{3+} and Mn^{4+} . This claim has never been confirmed nor refuted, but some measure of support comes from Russian workers (Levina and Robachevsky, 1955) who injected suspensions of MnO , MnO_2 , Mn_2O_3 and Mn_3O_4 of particle sizes less than $3\ \mu m$ intratracheally into rats. It was observed that the higher oxides were more toxic.

Although Mn neurointoxication is primarily an occupational health hazard, non-occupational cases of Mn poisoning have also been reported. The most comprehensive study is that of Kawamura et al. (1941) which concerns a Japanese family who maintained a bicycle-repair shop. At one time, they buried over 300 old dry-cell batteries from their shop near a well which supplied water for the family. Later, six members of the family and ten neighbors developed symptoms of a neurological variety including hypertonic muscles, mask-like faces, painful, rigid joints, tremor in the arms, and hypersalivation. Mental disturbances were also observed. Eventually, three died, one of suicide, and severe pathological changes in brain tissue were noted at autopsy. These changes were especially prominent in the globus pallidus. The survivors had elevated levels of Zn and Mn in their blood and urine. The Mn concentration of the well water during the time of intoxication was estimated to have been 20-30 mg/l.

A second report of possible Mn poisoning of a non-occupational variety comes from Banta and Markesbery (1977) who described a patient with both extrapyramidal signs and dementia. He was 55 years of age, had worked for two months in a steel mill some 30 years earlier, and had been consuming large doses of mineral tablets for about five years before being admitted to hospital. Brain biopsies revealed morphological evidence of presenile dementia, including numerous neuritic plaques and neurofibrillary tangles; Mn levels were elevated in biopsied tissue, serum, hair, and urine. The investigators could not resolve the relationship between the clinical picture, the elevated Mn levels, and the neuropathological findings. They concluded that their patient may have had two separate disorders: 1) Alzheimer's disease, and 2) chronic Mn intoxication from the ingestion of high doses of mineral supplements.

It is important to address the question of whether or not chronic Mn intoxication is truly a form of Parkinsonism. The similarity between Mn poisoning and paralysis agitans (Parkinson's disease) was first pointed out by Couper (1837) based on his clinical findings and this comparison was further developed by more contemporary researchers, particularly Mena and Cotzias' group. Following the success of L-dopa therapy in Parkinson's disease, they (Mena et al., 1970) used this drug with similar benefit in patients with chronic Mn poisoning. Of their six patients, five showed significant improvements including decreased rigidity, increased postural stability, and less facial masking. Rosenstock et al. (1971) confirmed their results by showing that high doses of L-dopa could alleviate the rigidity and bradykinesia caused by Mn intoxication. Shortly thereafter, Hornykiewicz (1973) reported

reductions in striatal dopamine levels at autopsy of a man who had suffered from chronic Mn poisoning, and expressed the view that "striatal dopamine deficiency is characteristic of the Parkinsonian syndromes of any etiology: idiopathic, postencephalitic, senile-arteriosclerotic, as well as the condition produced by chronic manganese poisoning and neuroleptic drugs." Further support for the "Parkinsonian" hypothesis of chronic manganism comes from experimental animal studies (detailed in Section 1.3.2.3) which have demonstrated changes in the levels of dopamine and its metabolites in response to chronic Mn administration. Emphasizing the similarities between manganism and Parkinson's disease, Donaldson et al. (1980) have speculated on the possible mechanism by which Mn exerts its selective CNS toxicity. Extending the work of Gillette et al. (1955) and Kappus and Schenkman (1978) who documented the ability of Mn^{2+} to stimulate the autoxidation of catecholamines, Donaldson et al. (1980) have shown Mn^{2+} to be much more effective than Cu^{2+} , Ni^{2+} , Zn^{2+} , Mg^{2+} or Ca^{2+} in its ability to enhance the autoxidation of dopamine and the concurrent production of H_2O_2 . They suggested that, in Mn intoxication, dopaminergic nerve cell degeneration might be induced in vivo as a result of free radicals produced during dopamine autoxidation potentiated by Mn, and that such degeneration might be mediated by increased lipid peroxidation initiated by the increased levels of free radicals. Independent evidence for this hypothesis is lacking, however, since Mn^{2+} added to brain homogenates in vitro inhibits lipid peroxidation of membranes (Baishayee and Balasubramanian, 1971; Heikilla

and Gabbat, 1983), and brain tissue from Mn-treated rats has a lower lipid peroxidation potential than that of unexposed animals (Shukla and Chandra, 1981).

There are many indications that Mn poisoning may not be primarily a form of Parkinsonism. The leading exponent of this view has been Barbeau who presented an extensive review of the earlier literature (Barbeau et al., 1976) and concluded that, in variable degrees, all victims of Mn intoxication show some manifestations of dystonia, defined as a postural instability of complementary muscle groups, and that the syndrome is quite different from Parkinson's disease in a number of ways. Clinically, for example, the intention tremor seen in Mn poisoning is of the opposite sort to that seen in Parkinson's disease, which is a resting tremor that disappears with activity. The tremor of manganism; however, resembles that seen in Wilson's disease. The pathological findings in the brain also differ markedly from the classical findings in Parkinson's disease where depigmentation and loss of cells in the substantia nigra and locus coeruleus with little observable damage to the caudate-putamen or pallidum are characteristic (Alvord et al., 1974) (see Fig. 1.2 for a diagrammatic representation of the major brain nuclei involved in the extrapyramidal motor system). In manganism, on the other hand, pallidal degeneration is almost invariably stressed, along with damage to the caudate-putamen, but little or no change in the appearance of the substantia nigra has been noted (Ashizawa, 1927; Canavan et al., 1934; Trendtel, 1936; Stadler, 1935-36; Voss, 1939; Kawamura et al., 1941; Parnitzke and Peiffer, 1954). Only one report, that of Bernheimer et al. (1973), has mentioned major involvement of the substantia nigra, that in the form of spotty

degeneration. Pentschew (1966) has expressed a similar view to that of Barbeau. Based on the neuropathological findings in humans, as well as his own experimental work with Mn administration in monkeys (Pentschew et al., 1963), he suggested that Mn encephalopathy, kernicterus, postictic encephalopathy, idiopathic torsion dystonia, and Hunt-van Bogaert's progressive pallidum-subthalamic nucleus atrophy form a disease group related to selective damage of the pallidum-subthalamic nucleus system. Clinically and neuropathologically, this group of diseases is rather similar to Wilson's disease and differs fundamentally from Parkinson's disease which is characterized by a dying-off of the pigmented nigrostriatal dopaminergic neurons. It should also be noted that in Parkinson's disease, it is necessary that 80-90% of the nigrostriatal tract be destroyed before the typical clinical manifestations of the disease become evident (Bernheimer et al., 1973), thus depigmentation in the substantia nigra is usually quite obvious at autopsy. As pointed out above, this has not generally been observed in chronic Mn intoxication. In addition, it has not always been possible for investigators to confirm the usefulness of L-dopa therapy in chronic Mn poisoning. In contrast to the success of Mena et al. (1970), Greenhouse (1971) and Cook et al. (1974), were unable to effect any significant improvements in their patients (a total of 7) with up to 8 g/day of L-dopa. Cook et al. (1974) deduced that a favorable response to L-dopa was dependent upon the presence of rigidity and/or dystonia as major clinical signs. None of their patients, nor those of Greenhouse (1971) were rigid or dystonic, but those patients of Mena et al. (1970) and that of Rosenstock et al. (1971) who responded to L-dopa were rigid and/or dystonic. Thus, it must be concluded that manganism is a complex

syndrome of extrapyramidal motor dysfunction which, although it may, during certain stages of its development, share some similarities with Parkinson's disease, is probably not strictly a form of Parkinsonism.

In addition to L-dopa therapy, treatment of chronic Mn intoxication with chelating agents, particularly EDTA, has been attempted. Generally, improvements have been non-existent or slight. Poor responses were especially prevalent in patients who had been afflicted with the disorder for several years (Cook et al., 1974; Wynter, 1962). . . Penalver (1957), however, reported significant improvements in a miner with manganism after EDTA treatment. The development of effective chelating agents may be of importance, therefore, especially for the treatment of mild forms or the early stages of Mn intoxication.

1.3.2.3 Experimental Mn Intoxication

Many animal studies have been conducted in order to investigate the neuropathological, neurochemical and/or neurobehavioral correlates of chronic Mn intoxication. In addition, the toxic effects of Mn on organs other than the brain have also been studied.

Many of the earlier animal experiments were aimed at producing behavioral changes of an extrapyramidal nature in animals that would mimic or at least show similarities with the clinical findings of human Mn intoxication, and then correlating these behavioral changes with pathological lesions in the brain. One of the first such studies

is that of Mella (1924) who administered $MnCl_2$ by injection to rhesus monkeys for 18 months. He observed a progression of neurological signs of intoxication in his Mn-treated monkeys beginning with chorea (muscular twitching) and athetosis (involuntary muscular distortion of the limbs) which later evolved into muscular rigidity, tremor of the hands, and contracture of the fingers. Histological examination revealed marked degenerative changes in the striatum and globus pallidus and, in addition, necrosis of the liver.

Van Bogaert and Dallemagne (1945-46) compared two routes of administration of Mn - inhalation and oral - with regard to their respective abilities to produce neurological impairment in rhesus monkeys. One monkey received daily 1 hr exposures to MnO_2 -containing dust for 100 days, while a second was given 10-15 mg/day $MnSO_4$ in its food for 300 days. The animal exposed by inhalation developed ataxia, an intention tremor, and later paralysis of the legs, whereas the animal exposed orally did not develop movement disorders. Histological examination of the brain of the affected monkey did not reveal selective damage to the globus pallidus, rather changes were scattered throughout the CNS and included damage in the cerebellum and spinal cord. This study resulted in the hypothesis that only pulmonary exposure to Mn was capable of producing the neurological syndrome typical of chronic Mn poisoning. This view, however, must be considered false since other researchers have successfully produced extrapyramidal dysfunction in primates by administering Mn via injection (Mella, 1924; Pentschew et al., 1963; Neff et al., 1969), and Kawamura et al. (1941) documented the occurrence of Mn poisoning in humans due to excessive exposure via the drinking water. The differences noted by van Bogaert and Dallemagne in

their two monkeys might be accounted for by a greater absorption and retention of Mn when administered by inhalation than when administered orally (see Section 1.1.5).

Pentschew et al. (1963) have given a very detailed description of the neuropathology in a rhesus monkey intoxicated by intramuscular injections of MnO_2 suspended in olive oil (5500 mg MnO_2 total dose). Nine months after initiation of the treatment, symptoms of an extrapyramidal nature began to develop including dystonic flexing of the arms and legs, hyperexcitability, and a general clumsiness while moving. Histopathologically, severe selective damage to the medial pallida and subthalamic nuclei was the major finding. Later, Pentschew (1963) described athetoid, dystonic posturing of the fingers and arms of chimpanzees intoxicated with Mn.

Neff et al. (1969) reported the development in squirrel monkeys of muscular rigidity, dystonic postures of the extremities, and fine, rapid intention tremors 4 months after the subcutaneous injection of a suspension of 200 mg MnO_2 . Significantly, no neuronal nor glial abnormalities were noted during histological examination of the cerebral cortex, caudate, pallidum, thalamus, hypothalamus, hippocampus, subthalamic nucleus, substantia nigra and cerebellum, but a 50-55% decrease in the dopamine content of the caudate nucleus was noted. Norepinephrine levels in the cerebrum and brainstem were normal. This constitutes the first report of neurochemical changes in the absence of observable histological changes after chronic Mn exposure. More recently, Chandra et al. (1979) have reported decreases in dopamine,

norepinephrine and 5-HT in several brain regions of rhesus monkeys after 18 months of oral exposure to Mn. Neither neurobehavioral nor neuropathological parameters were included in that report.

It should be evident from the above survey of experimental Mn intoxication in primates that both the major behavioral deficits (athetoid, dystonic postures with an intention tremor) and the major neuropathological findings (destruction in the pallidum, sub-thalamic nuclei, and striatum) support the contention of Barbeau et al. (1976) that Mn poisoning bears no essential similarity to Parkinson's disease. Experimentally, marked cell loss of nigral dopaminergic neurons in conjunction with a lesion of the parvocellular part of the red nucleus in primates (an animal model of Parkinsonism) results in the combined appearance of hypokinesia, a typical Parkinsonian tremor, and, less predictably, rigidity in primates (Pechadre et al., 1976). These behavioral abnormalities, although extrapyramidal in nature, bear only a partial similarity to those typically observed after chronic Mn exposure. Furthermore, experimental depletion of striatal dopamine in primates by lesions in the substantia nigra consistently produces Parkinsonian hypokinesia (decreased motor activity) if and only if the lesion is severe enough to reduce the striatal dopamine content by over 80% (Poirier et al., 1966). The 40-55% depletion in caudate dopamine reported by Neff et al. (1969) and Chandra et al. (1979) after chronic Mn exposure cannot be considered as the essential or primary biochemical lesion leading to the neurological expression of Mn intoxication since, by itself, a 50% depletion of striatal dopamine should result in no observable extrapyramidal motor dysfunction.

It has not been possible to produce definite extrapyramidal movement disorders in experimental animals other than primates in response to chronic Mn exposure. However, numerous investigations have been carried out on rats, mice, and rabbits in order to reach a clearer understanding of the important histological, biochemical, and neurochemical effects of Mn intoxication. These studies will now be summarized.

In adult rats receiving ~3.5 mg Mn/kg ip, histological changes in the brain did not occur until after 100 days of exposure (Chandra and Srivastava, 1970), at which time scattered neuronal degeneration in the cerebral and cerebellar cortices began to be observed. By 180 days, a significant number of degenerated neurons with pyknotic nuclei were evident, as well as marked swelling of oligodendrocytes. The basal ganglia, however, appeared normal, as did the meninges, choroid plexus and blood vessels. In young rats (21 days old), similar effects have been documented in response to chronic Mn exposure, but the pathological changes were seen to occur much sooner than in the adult. Significant neuronal degeneration in the cerebral cortex and cerebellar cortex was evident by 45 days, with involvement of the caudate nucleus by 60 days (Chandra and Shukla, 1978). Thus, Mn exposure produced morphological alterations sooner, and more brain regions were involved, in young rats than in adults, indicating that the developing mammalian brain may be more susceptible to Mn poisoning than that of the adult. After 25 days of ip injections of 6 mg Mn/kg to adult rats, enzymatic changes in the brain were observed in the absence of visible histological changes (Singh et al., 1974). The activities of acid- and alkaline-phosphatase, lactic dehydrogenase, and succinic

dehydrogenase were decreased by 20-50% in Mn treated rats, whereas ribonuclease and ATP-ase were unchanged. The authors interpreted these results as an indication that Mn interferes with energy metabolism. Similar decreases in the activities of succinic dehydrogenase, ATP-ase, and adenosine deaminase were noted in the brains of suckling rats exposed for 30 days to Mn through the milk of nursing dams receiving 15 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ /kg/day orally. Both the decrease in enzyme activities, and the increase in cerebral Mn content, were greater in the pups than in the mothers, again indicating that young mammals are more susceptible to the toxic effects of Mn than adults. This may be a consequence of the fact that neonates absorb and retain more Mn than adults. In fact, the ability to excrete Mn does not develop in rodents before 18 days of age. Until that time, they show an absence of elimination of injected, ^{54}Mn (Cotzias et al., 1974).

In the rabbit, intratracheal inoculation of MnO_2 (400 mg) produced lethargy and paralysis of the hind limbs after 18-20 months. In the cerebral cortex, particularly the parietal cortex, a mild to moderate depletion of nerve cells was noted. Moderate to severe loss accompanied by astrocytic proliferation and swollen oligodendrocytes was observed in the caudate putamen, whereas thalamic, hypothalamic, subthalamic and red nuclei appeared normal. There was mild neuronal loss in the substantia nigra. Enzyme histochemistry revealed decreases in neuronal acid phosphatase and ATP-ase. Alkaline phosphatase activity, which was localized in capillary and vascular endothelium, was unchanged, indicating that the toxic effects of Mn were prominent in neurons and not in other cells of the brain.

Since the discovery by Neff et al. (1969) of decreased levels of dopamine and 5-HT in the brains of Mn-intoxicated monkeys, several investigations have been undertaken to characterize more fully the effects of Mn exposure on central neurotransmitters, particularly dopamine (DA) and norepinephrine (NE) (see Fig. 1.1 for a diagrammatic representation of the major biochemical pathways of dopamine in the brain). In rabbits inoculated intratracheally with 400 mg MnO_2 , whole-brain DA was decreased by 21%, NE by 58%, and 5-HT was unchanged after 24 months of exposure (Mustafa and Chandra, 1971). Studies in rats and mice have demonstrated that the response of central catecholamine levels to Mn exposure is biphasic in nature. Administration of Mn for two months or less results in either no change or an increase in the levels of NE, DA, and homovanillic acid (HVA), a major metabolite of DA (Chandra et al., 1979a,b; Gotzias et al., 1974; Shukla and Chandra, 1979; Chandra and Shukla, 1981a,b). If increases are observed, they are 15-30% above control levels. This early phase of Mn intoxication is also characterized by an increased activity of monoamine oxidase (MAO) (Chandra and Shukla, 1978; Seth et al., 1977; Shukla and Chandra, 1976); an increased rate of synthesis of DA and NE (Chandra and Shukla, 1981b) and an increased turnover of striatal DA (Shukla and Chandra, 1981). The activity of striatal and hypothalamic tyrosine hydroxylase (TH), the rate limiting enzyme in the synthesis of DA and NE, has been found to be unaffected by the addition of Mn in vitro, indicating that the increased synthesis of catecholamines seen after subchronic administration of Mn is not due to a direct activation of TH by Mn.

Administration of Mn for more than four months results in a 20-40% decrease in the levels of DA, NE, and HVA compared to controls (Bonilla and Diez-Ewald, 1974; Chandra and Shukla, 1981a; Autissier et al., 1982). During this phase, MAO activity is normal in all-brain regions; DA turnover in the corpus striatum is decreased, and NE turnover is increased in the brainstem but decreased in the hypothalamus. Normal levels of DA can be re-established by L-dopa administration (Bonilla and Diez-Ewald, 1974).

The effects of chronic Mn exposure on other CNS neurotransmitters have also been studied in experimental animals. Levels of 5-HT were decreased (Shukla and Chandra, 1979; Kimura et al., 1978) or unchanged (Chandra et al., 1979a) in "whole brain" rodent studies. However, primate studies have revealed that 5-HT levels may be decreased in some brain regions (caudate, midbrain, medulla) but unaltered in others (Chandra et al., 1979c; Neff et al., 1969) after long-term exposure to Mn.

The effects of Mn on GABA-ergic parameters are not well studied and are somewhat controversial. Bonilla (1978) reported a 77% increase in the striatal GABA content of rats consuming drinking water containing 10 mg $MnCl_2$ /ml. The activities of neither the major synthetic enzyme, glutamic acid decarboxylase (GAD), nor of the major catabolizing enzyme, GABA-transaminase (GABA-T), were altered. On the other hand, Chandra et al. (1982) reported that daily ip injections of 6 mg Mn/kg to rats for 30 days resulted in 24-30% reductions in whole brain GABA content, and in GAD and GABA-T activities. Such contradictory findings are difficult to resolve, especially since increase in Mn content was about equal in the two studies (60-70% above

control levels). Unfortunately, concurrent estimations of the concentrations of other neurotransmitters were not performed in either of these studies.

Changes in cholinergic parameters in response to Mn administration have also not been extensively studied. The only indication of possible cholinergic involvement is the finding that cerebral acetylcholinesterase (AChE) activity is decreased due to Mn administration in rats (Sitaramayya et al., 1974; Seth et al., 1977).

Neuroleptic drugs such as haloperidol, spiroperidol and chlorpromazine, which are used in the treatment of schizophrenia, occasionally cause extrapyramidal motor disorders in patients being treated with these drugs (Ezrin-Waters et al., 1981; Kolata, 1979). This is probably a result of the fact that neuroleptics are potent DA-receptor blockers (Seeman et al., 1974; Seeman et al., 1976). It is because of this ability to block DA receptors that unilateral microinjection of neuroleptics into the striata of experimental animals causes ipsilateral turning behavior, that is, turning towards the side of the striatum being stimulated (Cobls, 1973; Ungerstedt et al., 1969) and bilateral injections cause a freezing, cataleptic response (Costall et al., 1972). Interestingly, the intrastriatal microinjection of Mn^{2+} (25-50 μg) to rats causes a similar behavioral pattern, that is, a predominant ipsilateral turning behavior which continues for up to 30 minutes (Inque et al., 1975). This effect is fairly specific for Mn^{2+} . Other metals do not produce turning, or cannot be administered in high enough concentrations to be tested since they cause convulsions (Cu^{2+} , Zn^{2+}). Thus, the possibility arises that chronic Mn exposure may inactivate postsynaptic DA-receptors in the corpus striatum, or may

interfere with the ability of DA to properly bind to these receptors, or may inhibit the release of DA from the presynaptic nerve terminals. No detailed studies examining these possibilities have been reported in the literature, thus further investigations are required to determine the influence of Mn on DA-receptors.

Another phenomenon linking Mn and dopaminergic transmission is the finding that intraventricular administration of Mn^{2+} stimulates prolactin secretion, a process which is under central dopaminergic control (Barbeau et al., 1976). In this regard, the effect of Mn is again similar to that of the neuroleptic drugs (Friesen et al., 1972). On the other hand, Ni^{2+} specifically inhibits prolactin release (LaBella et al., 1973), indicating that the effect of Mn^{2+} is not a general effect of heavy metal cations.

In addition to its effect on the CNS, the chronic administration of Mn to experimental animals has also been shown to cause pathological changes in other organs. The major reported effects are: 1) progressive degeneration of the seminiferous tubules of the testes, accompanied by a corresponding decline in testicular succinic dehydrogenase and ATP-ase activity (Seth et al., 1973), and 2) focal necrosis of liver parenchyma, also accompanied by reduced succinic dehydrogenase activity (Singh et al., 1974).

FIGURES

Figure 1.1

Major metabolic pathways of dopamine in the CNS.

Abbreviations: L-dopa, L-dihydroxyphenylalanine; COMT, catechol-O-methyltransferase; MAO, monoamine oxidase; DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid; MHPG, 3-methoxy-4-hydroxyphenylethyleneglycol; VMA, vanilmandelic acid.

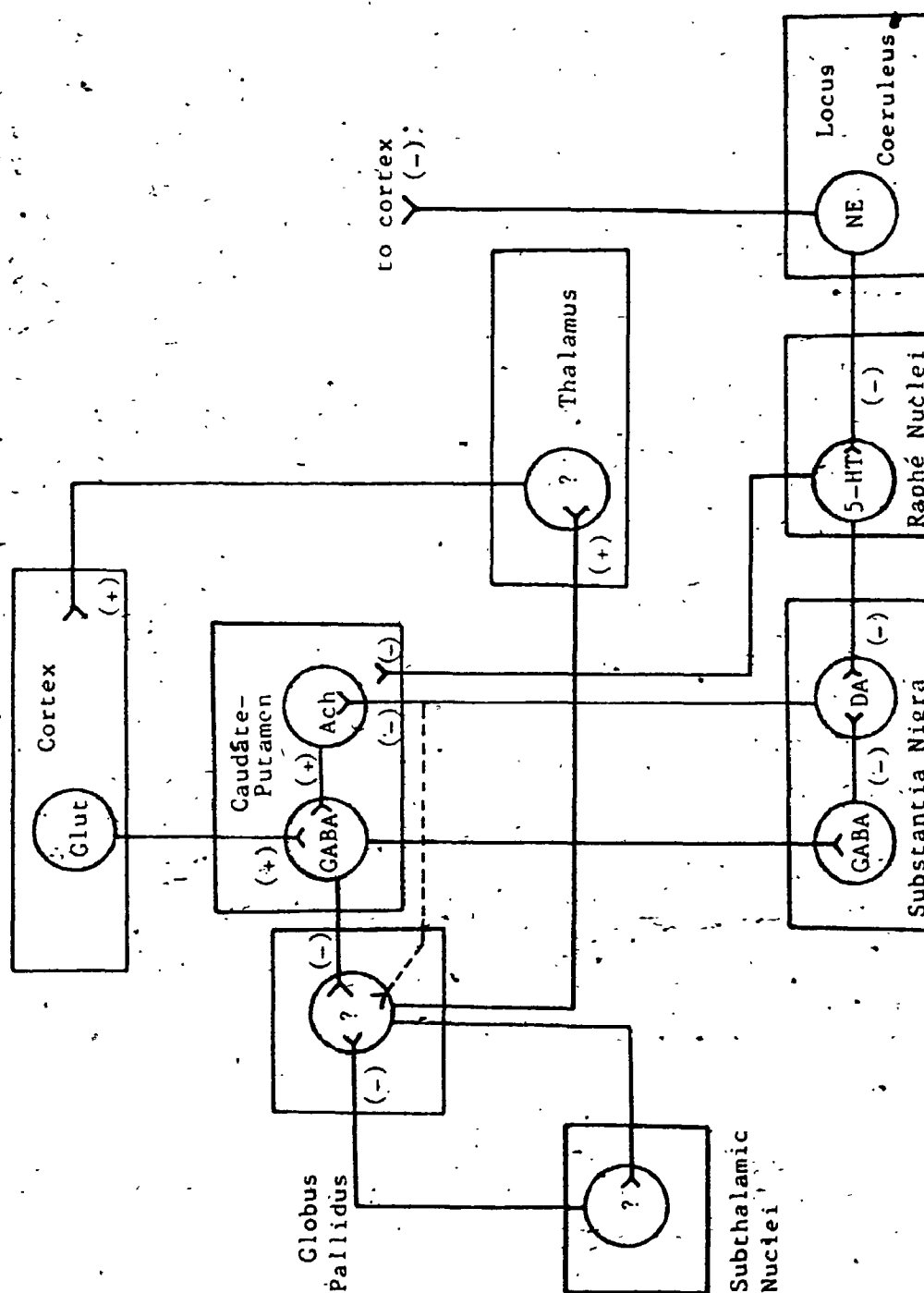


Figure 1.2

Major nuclei of the extrapyramidal motor system.

Abbreviations: Glut, glutamate; GABA, γ -aminobutyric acid; Ach, acetylcholine; DA, dopamine; 5-HT, 5-hydroxytryptamine; NE, norepinephrine; ?, unknown; (+), stimulatory; (-), inhibitory. Adapted from Antelman and Caggiula, 1977; McGeer et al., 1977; Kim, 1978; Pycock, 1978.

The raphe nuclei and locus coeruleus are not generally considered to be part of the extrapyramidal system, but they are depicted here due to their neuronal connections with nuclei of the extrapyramidal system.

CHAPTER TWO

AIMS AND OBJECTIVES

Since Mn is both an essential trace element and a neurotoxicant which, under suitable conditions of exposure, can cause a debilitating neurological syndrome of extrapyramidal motor dysfunction, investigations dealing with the absorption, plasma binding, tissue distribution, subcellular binding, excretion, and the effects of Mn on various metabolic processes all have relevance to both the normal metabolism as well as the toxicology of Mn. A survey of the literature has revealed several areas in which further study is necessary. The following is a list of specific objectives to be met in the present series of investigations:

- 1) to characterize the tissue distribution and excretion of Mn^{2+} administered as a single dose and to investigate some factors, such as the presence or absence of known metal-binding proteins, which might influence the kinetics of Mn.

- 2) to examine the binding of Mn to plasma proteins in order to determine whether transferrin, albumin, or some other ligand is the major Mn-binding protein in blood.

- 3) to determine the pattern of accumulation of Mn in tissues during chronic administration.

- 4) to determine the pattern of regional accumulation of Mn in the brain.

- 5) to determine the effects of Mn accumulation on the normal tissue concentrations of other essential trace elements.

6) to determine whether chronic Mn administration causes significant histological changes in tissues and to characterize the nature of these changes.

7) to examine the natural elimination rate of excess Mn and to determine the usefulness of EDTA to remove excess Mn from tissues.

8) to determine the regional effects of chronic Mn administration on the biosynthesis and biodegradation of the neurotransmitters DA, NE, and 5-HT.

9) to determine the effects of Mn on striatal DA receptors.

10) to compare the organ distribution, accumulation, and effects of Mn administered as ip injections vs orally in drinking water.

CHAPTER THREE

THE DISTRIBUTION, EXCRETION, AND PLASMA BINDING OF Mn

3.1 Methods

3.1.1 Animals

Male Sprague-Dawley rats (150 to 250 g), obtained from Canadian Breeding Farm and Laboratory, St. Constant, Canada, were used for the experiments. They were housed in metal cages with free access to rat chow (Purina Rat Chow) and tap water for several days before the experiments were performed.

3.1.2 Reagents

Carrier-free $^{54}MnCl_2$ and L- $[^3H]$ dopa (5-15 Ci/mmol) were obtained from New England Nuclear Corporation (Lachine, Quebec). Nonradioactive manganese ($MnCl_2 \cdot 4H_2O$), zinc ($ZnSO_4 \cdot 7H_2O$ or $Zn(C_2H_3O_2)_2 \cdot 2H_2O$), Fe ($FeCl_2 \cdot 4H_2O$), and Cu ($CuSO_4 \cdot 5H_2O$) were obtained from Fisher Scientific Company (Fair Lawn, N.J.) and L-dopa (L-3,4-dihydroxyphenylalanine) from BDH Chemicals (Toronto, Canada). Transferrin (human) and albumin (rat) were obtained from Sigma (St. Louis, MO).

3.1.3 Surgical Procedure

Rats were anesthetized with pentobarbital, and the common bile duct and jugular vein were cannulated with polyethylene tubing as described previously (Cherian and Vostal, 1977). A heating pad was used to maintain body temperature at 37°C over the course of the experiment. A tracheal tube was inserted to ensure the free passage of air during the collection period and additional pentobarbital was administered if necessary. Bile was collected for a control period of 30 min to establish a constant flow after which test solutions were administered iv via the jugular vein, and bile samples were collected for an experimental period of 3 hr at 0.5-hr intervals. Fluid volume lost by the output of bile was replaced periodically by infusing normal saline through the jugular vein. Rats were killed by aortal exsanguination. Tissues (blood, heart, lungs, liver, spleen, kidneys, pancreas, testes, brain, duodenum, and bone) were removed immediately and kept chilled on ice until counting. Urine was removed from the bladder with a syringe.

3.1.4 Radioactivity Measurements

⁵⁴Mn in bile, urine, and tissues was measured with a Packard gamma scintillation spectrometer. ³H was measured in a Packard liquid beta scintillation counter after appropriate sample preparation (Cherian, 1978).

3.1.5 Experiment 1: Effect of Dose

Rats whose bile ducts and jugular veins had been cannulated were injected iv with 0.1, 1.0, or 2.0 mg Mn/kg as $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ labelled with carrier-free ^{54}Mn (10 $\mu\text{Ci/kg}$ body wt.). Bile, urine, and tissue samples were subjected to gamma counting to determine the distribution of Mn. Bile was fractionated on a Sephadex G-75 column to partially characterize the biliary binding of Mn.

3.1.6 Experiment 2: Effect of Zinc Pretreatment

Rats were pretreated ip with either Zn (10 mg/kg as $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) or an equal volume of 0.9% NaCl, 24 hr prior to cannulation of the bile duct and jugular vein. Mn (1.0 mg/kg) labelled with ^{54}Mn then was administered iv via the jugular vein. Bile was collected for 3 hr after which animals were killed. The biliary excretion of ^{54}Mn and the binding of ^{54}Mn in the liver cytosol were compared between control and Zn-pretreated rats. Liver tissue was homogenized in 4 vol of TKM-0.25 M sucrose buffer, and supernatant fractions were prepared by ultracentrifugation (105,000g for 1 hr at 4°C). The supernatant fluid was then fractionated on a Sephadex G-75 column, and the distribution of ^{54}Mn was determined by gamma counting.

3.1.7 Experiment 3: Interaction with L-dopa

Solutions of L-dopa alone, Mn alone, or a solution of Mn + L-dopa were prepared in 0.9% NaCl under slightly acidic conditions (pH 5), at an Mn:L-dopa molar ratio of 1:1. The actual dosages administered were 1.2 mg/kg Mn, 4.1 mg/kg L-dopa, or a combination of 1.2 mg/kg Mn + 4.1 mg/kg L-dopa. Since it was of interest to follow the excretion of both Mn and L-dopa, four groups of rats were used. Group 1 received Mn (1.2 mg/kg) + ^{54}Mn ; Group 2 received L-dopa (4.1 mg/kg) + L- ^3H dopa; Group 3 received Mn + ^{54}Mn + L-dopa; and Group 4 received Mn + L-dopa + L- ^3H dopa. Injections were iv via the jugular vein. Bile was collected for 3 hr at which time rats were killed and organs removed.

3.1.8 Experiment 4: Binding of $^{54}\text{Mn}^{++}$ and Other Heavy Metal Ions to Transferrin and Albumin

Typically, 5 mg of albumin or transferrin were dissolved in 0.2 ml of 10 mM HEPES buffer, pH 7.4, or 10 mM Tris, pH 8.6, and incubated in the presence of $^{54}\text{Mn}^{2+}$, $^{59}\text{Fe}^{3+}$, $^{109}\text{Cd}^{2+}$, or $^{54}\text{Zn}^{2+}$ (30,000-40,000 cpm) for 1/2 hr at room temperature. The samples were then fractionated on Sephadex G-75 gel filtration columns eluted with either 10 mM HEPES, pH 7.4, or 10 mM Tris, pH 8.6. The fractions were collected and counted on a gamma counter.

3.1.9 Experiment 5: Binding of ^{54}Mn in Human and Rat Plasma

Blood was collected in heparinized syringes from the median cubital vein of normal, healthy human volunteers, and from the abdominal aorta of anesthetized rats. Plasma was prepared by centrifugation at 1,000xg for 10 min. Aliquots of plasma (generally 0.2 ml) were incubated in the presence of carrier-free ^{54}Mn (0.1 μCi) with or without preincubation in the presence of excesses of other metals (Fe, Zn, Cu) for 1/2 hr at room temperature. Incubations were carried out in 10 mM Tris buffer at pH 8.6 or 6.0, after which the incubates were applied on Sephadex G-75 columns and eluted with 10 mM Tris buffer, pH 8.6. Fractions were collected and counted for gamma radiation, and the total metal content of the fractions was measured by atomic absorption spectrophotometry. (See Section 4.1.3 for details of metal estimation by spectrophotometry.)

3.1.10 Statistical Analysis

A variety of statistical tests were employed in the present series of experiments including two-way analysis of variance, Student's t-test, regression, and analysis of covariance. The Results section provides appropriate explanation as to which tests were used to analyze the data. Whenever statistical analyses were performed on data expressed as percentages or proportions, the arcsin transformation was employed to

ensure that data were normally distributed. The references for statistical methodology were Snedecor and Cochran (1967) and Sokal and Rolf (1969).

3.2 Results

3.2.1 Experiment 1

The biliary excretion of ^{54}Mn after the administration of 0.1, 1.0, and 2.0 mg Mn/kg is shown in Fig. 3.1. Excretion of the tracer was always greatest over the first half-hour after injection. As the dose of injected Mn was increased, progressively more of the administered dose (AD) was excreted in the bile over the 3-hr experimental period. A 3-hr total of only 27% AD was excreted when 0.1 mg/kg was administered. This value increased to 42% when 1.0 mg/kg was given, and to 51% when 2.0 mg/kg was injected. Urinary excretion was negligible for all three doses of Mn (0.01 to 0.03% AD). A high proportion of the Mn which had not been excreted by the end of the experimental period was localized in the liver (Figs. 3.2 and 3.3).

^{54}Mn excreted in bile was primarily bound to low-molecular-weight substances (Fig. 3.4). The in vitro addition of ^{54}Mn to control rat bile revealed a similar pattern of binding.

3.2.2 Experiment 2

Pretreatment with Zn altered neither the biliary excretion (Fig. 3.5) nor the binding of ^{54}Mn in the liver cytosol (Fig. 3.6). In the liver supernatant fraction, Mn was bound to high- and low-molecular-weight proteins, none to MT. This finding indicates that, unlike some other metals including Cd, Cu, Ag, and Zn, Mn does not bind to pre-synthesized MT in the liver.

3.2.3 Experiment 3

The administration of a solution of L-dopa + Mn significantly altered the normal distribution and biliary excretion of the metal. There was a significant decrease in the biliary excretion of Mn even though the bile flow remained unchanged (Table 3.1; Fig. 3.7).

Two-way analysis of variance revealed significant effects on the biliary excretion of Mn from both treatment ($F = 23.6$, $p < 0.001$) and time ($F = 46.2$, $p < 0.001$). Interaction between these two variables was insignificant ($F = 0.3$, n.s.). Figure 3.7 shows the computer-generated best fit exponential regression lines for the biliary excretion of Mn after injection of Mn alone or Mn + L-dopa. For the former, $r = -0.93$, $p < 0.01$; for the latter $r = 0.84$, $p < 0.01$. Standard linear regression analysis also produced statistically significant r values, but the data were found to be better represented by an exponential relationship. Although the % AD of Mn recovered in the bile was decreased in the

Mn + L-dopa group, there was no concurrent increase in the % AD in liver or other organs. This finding indicates that Mn, when administered with L-dopa, may be deposited to a greater extent in peripheral tissues which were not sampled such as skin, skeletal muscle, and connective tissues.

Mn produced no significant changes in the normal pattern of excretion of L-[^3H]dopa. As illustrated in Table 3.2, most of the recovered ^3H radioactivity was in the urine 3 hr after the administration of L-[^3H]dopa alone, a finding which is consistent with the results of a previous study (Coutinho et al., 1971) in which 50 to 70% of an oral dose of L-[^3H]dopa (50 mg/kg) administered to dogs was excreted in the urine within the first 24 hr postadministration. Of the tissues examined in the present study, the pancreas, kidney, and duodenum had the highest levels of radioactivity per gram of tissue. When only the retained radioactivity was considered, it was found that the highest proportion of the total retained ^3H was localized in the liver. The total radioactivity recovered from the urine and from the bile did not change significantly when Mn was administered along with L-dopa, nor was there a change in the ratio of urinary excretion versus biliary excretion (Table 3.3). Two-way analysis of variance revealed a significant effect on the biliary excretion of ^3H due to time ($F = 49.5$, $p < 0.001$) but differences due to treatment (L-dopa alone versus L-dopa + Mn) were insignificant ($F = 0.02$, n.s.). Figure 3.8 illustrates the biliary excretion of ^3H versus time. In addition to analysis of variance, these data were subjected to regression analysis. A significant time dependency was found for the biliary excretion of ^3H . For: L-[^3H]dopa alone, $Y' = -5.9X + 33.9$ ($r = -0.90$, $p < 0.01$); for

$L-[^3H]dopa + Mn$, $Y = -5.3X + 32.9$ ($r = -0.87$, $p < 0.01$). Analysis of covariance was employed to compare the slopes of the two regression lines, and an insignificant difference was found.

3.2.4 Experiment 4

Compared to the other heavy metal cations tested in the present study ($^{109}Cd^{2+}$, $^{65}Mn^{2+}$, $^{59}Fe^{3+}$), $^{54}Mn^{2+}$ was found to bind very poorly to purified albumin in aqueous solution at neutral (7.4) or basic (8.6) pH (Fig. 3.9). The addition of 6 mg exogenous albumin to a 100 μ l aliquot of rat plasma did not result in an increased level of ^{54}Mn radioactivity associated with the high molecular weight (HMW) proteins after subsequent incubation and fractionation on a G-75 column. This was so despite the fact that the protein content of the HMW fractions, as measured by the Lowry method, was more than doubled due to the added albumin. Also, the incubation of ^{65}Zn -albumin in the presence of excess Mn^{2+} (1 mM) did not result in the displacement of ^{65}Zn from the protein (Fig. 3.10).

In contrast to the results with albumin, ^{54}Mn was observed to become bound to transferrin, not as well as $^{59}Fe^{3+}$, but considerably better than $^{65}Zn^{2+}$ or $^{109}Cd^{2+}$ under the conditions of the present study (Fig. 3.11). Binding was more complete at a basic pH than under neutral conditions. Saturation of transferrin by preincubation in the presence of sufficient Fe (1.6 μ g Fe/mg protein) prevented the binding of subsequently added $^{54}Mn^{2+}$ (Fig. 3.12) indicating that Mn probably binds to the Fe sites on the protein if the protein is not saturated with Fe.

3.2.5 Experiment 5

The incubation of 200 μ l of human or rat plasma with 0.14 μ Ci ^{54}Mn at pH 6.0 or 8.6 followed by fractionation of the sample on a G-75 gel filtration column resulted in the association of the ^{54}Mn radioactivity with the HMW fractions (Fig. 3.13). Dialyzing the samples before application onto the column did not result in a lower recovery of radioactivity. Preincubating the plasma samples in the presence of 60 μ g of ^{55}Mn , however, greatly reduced the ^{54}Mn radioactivity subsequently associated with the HMW fractions (Fig. 3.13). Preincubation of aliquots of human (Fig. 3.14) or rat (Fig. 3.15) plasma in the presence of higher-than-saturating amounts of Fe, Zn, and Cu reduced the binding of subsequently added ^{54}Mn only in rat plasma at pH 8.6, otherwise the binding of ^{54}Mn to the HMW fractions revealed essentially the same profiles as in the absence of added metals.

3.3 Discussion

Papavasiliou et al. (1968) showed that various substances including L-dopa, dopamine, adrenaline, isoproterenol, glucagon, and cAMP could depress the biliary excretion of ^{54}Mn in rodents. This effect was related to the relative ability of the substance to increase the concentration of cAMP in the liver. Cotzias et al. (1972) later

suggested that, in addition, L-dopa might bind Mn thereby affecting the distribution of the metal. L-dopa has the ability to chelate several metals in vitro (Kikkawa et al., 1955; Rajan et al., 1976). However, in the present study, no evidence for the in vivo chelation of Mn by L-dopa was observed. Radioactivity from the administration of L-[³H]dopa was mainly excreted in the urine. Thus, if the chelation of Mn by L-dopa was an important factor affecting the distribution of the metal, it might be expected that the administration of a solution of L-dopa + Mn would have the effect of increasing the urinary excretion of Mn. Yet the coadministration of L-dopa + Mn did not cause an increase in the urinary excretion of ⁵⁴Mn which remained at the normal negligible levels, nor were the kidney levels of the metal increased. Similarly, Mn, which was excreted mainly in the bile, did not enhance the normal biliary excretion of L-[³H]dopa and its metabolites. This finding was so even though the solutions of Mn + L-dopa used in the experiment had been prepared under conditions which favored the in vitro formation of an L-dopa-metal chelate (Rajan et al., 1976). There is no doubt that L-dopa affects the distribution and biliary excretion of intravenously injected Mn, but this effect is probably not as a result of its ability to chelate metals.

In addition to the direct action of L-dopa on the liver, which has been described previously (Papavasiliou et al., 1968), the influence of L-dopa on the cardiovascular system may also contribute to its depressing effect on the biliary excretion of Mn. Dopamine in low doses has a slight peripheral vasodilating effect in several animal species (Burn and Rand, 1958; Holtz and Credner, 1942; McDonald and Goldberg, 1963). Also, dopamine increases cardiac output by increasing cardiac

contractile force (Tjandramaga et al., 1973). Not surprisingly, the effects of L-dopa, the immediate precursor of dopamine, are similar to those of dopamine itself (Goldberg and Whitsett, 1971) since L-dopa is readily decarboxylated to dopamine in the peripheral circulation. In addition, L-dopa may exert a central hypotensive effect (Henning and Rubenson, 1970) since, unlike dopamine, L-dopa is able to cross the blood-brain barrier. In the present study, increased cardiac contractile strength coupled with peripheral vasodilation may, in part, account for the decrease in Mn secreted into the bile when Mn + L-dopa were administered concurrently since a higher proportion of the injected iv dose of Mn might be diverted peripherally with the increase in peripheral blood flow. Thus, less Mn would reach the liver over the 3 hr of bile collection and since the biliary excretion of Mn is dose dependent (Fig. 3.1), the effect would be similar to that of simply injecting less Mn. Thus, the excretion of Mn may not be strictly a function of the dose per se, but a function of the amount of Mn that actually reaches the liver.

With regard to Mn and MT, others have shown that Mn administration does not induce the synthesis of MT (Piotrowski and Szymanska, 1976). In the present study, I have shown that this metal does not bind to hepatic MT which has been pre-synthesized by an injection of Zn. This finding is contrary to the behavior of Cd, an element whose rate of biliary excretion was much decreased after induction of MT synthesis by prior administration of Zn (Cherian, 1980). Differences between Mn and Cd, with regard to their binding ligands, undoubtedly contribute to the observed differences in their responses to the presence of MT. Histidine and cysteine bind metals more securely than other amino acids,

histidine through its imidazole nitrogen, and cysteine through its thiol group. Certain metal ions, including Fe^{3+} and Mn^{2+} , have higher affinities for imidazole and tend to form coordination compounds with proteins whereas some other metals such as Zn^{2+} , Cd^{2+} , and Cu^{2+} reveal higher affinities for thiol and form covalent linkages with proteins (Luckney and Venugopal, 1977). MT has a high proportion of cysteine residues ($\sim 30\%$) but it is virtually devoid of histidine (Bremner and Davies, 1975; Winge et al., 1975). This finding may explain the inability of MT to affect the kinetics of Mn.

There is disagreement in the published literature concerning the nature of Mn-binding ligands in serum and plasma. Foradori et al. (1967) reported that ^{54}Mn became bound almost exclusively to a β_1 -globulin and Cotzias and Bertinchamps (1960) had previously stated that this β_1 -globulin was not transferrin since the protein-bound ^{54}Mn did not exchange with Fe. These researchers hypothesized the existence of a specific Mn-transport protein in plasma (transmanganin). Partial support for this contention was provided by Himmelhoch et al. (1966) who found that virtually 100% of Mn in human serum was associated with a fraction that contained only about 15% of the total serum Fe. If it can be assumed that most of the Fe in serum is bound to transferrin, then it thus may be that Mn is not bound to transferrin in human serum. The results of the present study also lend support to the possibility that ligands exist in human plasma which have a substantially higher affinity for Mn than for Fe, Zn or Cu. It was reasoned that if transferrin was the primary Mn-binding protein in plasma, then the addition of a saturating amount of Fe to an aliquot of plasma should result in a significant decrease in the level of ^{54}Mn radioactivity which would

subsequently become associated with the HMW fractions. It was further assumed that if excess Zn^{2+} and Cu^{2+} were also added to the aliquots of plasma, then it might be possible to completely saturate all the specific (e.g. transferrin) and non-specific (e.g. albumin) heavy metal binding sites and thus to completely prevent the binding of subsequently added ^{54}Mn . But the presence of large excesses of these other metals did not abolish the binding of ^{54}Mn to the HMW fractions of human plasma. In rat plasma, on the other hand, preincubation in the presence of Fe, Zn and Cu at pH 8.6 significantly reduced the binding of subsequently added ^{54}Mn compared to that in the absence of competing metals. This is in accord with the proposal of Keefer et al. (1970) that transferrin is the major Mn-binding ligand in rat serum. However, the present study also demonstrates the influence of pH on the binding of ^{54}Mn to plasma proteins. At neutral and acidic pHs, divalent Mn is extremely stable, but at basic pHs the oxidation of Mn^{2+} to Mn^{3+} is favored. That this process actually occurs can be verified visually by dissolving $MnCl_2$ in Tris buffer at pH 8.6. The original solution is clear and virtually colourless, but through time it becomes progressively browner as Mn^{2+} is oxidized to Mn^{3+} . Thus, if it can be assumed that under basic conditions, much of the dissolved $^{54}Mn^{2+}$ is converted to the trivalent form, then in rat plasma, preincubation in the presence of Fe, Zn and Cu prevents the binding of subsequently added Mn^{3+} (pH 8.6) but not Mn^{2+} (pH 6.0), whereas in human plasma, competing metals can prevent the binding of neither Mn^{3+} nor Mn^{2+} . Thus, there are differences in the plasma-binding of Mn due to species as well as to the valence state of the metal. Cotzias and Papavasiliou (1962) maintained that the valence state of endogenous Mn in human serum and

cerebrospinal fluid was Mn^{3+} . If this is true, and if Mn^{3+} is assumed to be bound primarily to transferrin in human plasma, then the presaturation of plasma with Fe (plus Zn and Cu) at pH 8.6 should have virtually abolished the binding of ^{54}Mn to HMW plasma proteins. That this did not occur is suggestive of the presence in human plasma of Mn-binding ligands other than transferrin. In any case, the results of the present investigation indicate that albumin is almost certainly not a major Mn-binding ligand in plasma, contrary to the suggestion of Nandedkar et al. (1973).

Table 3.1

The Effect of L-dopa on the Distribution and Excretion of Manganese

The ^{54}Mn content of urine, bile, and other tissues (blood (%/10 ml), bone (%/g), heart, lungs, kidney, spleen, duodenum, testes, and brain), expressed as % of administered dose and as % of total recovered counts three hours after the iv injection of solutions of Mn alone (1.2 mg Mn/kg + 10 μCi ^{54}Mn /kg), or Mn + L-dopa (1.2 mg Mn/kg + 10 μCi ^{54}Mn /kg + 4.1 mg L-dopa/kg). Values are means \pm S.D., n = 6.

*Volume is expressed as ml bile/rat/3 hr collection period.

	Mn CONTENT			
	% Administered Dose		% Total Recovered Counts	
	Mn alone	Mn + L-dopa	Mn alone	Mn + L-dopa
Urine	0.03 \pm 0.02	0.02 \pm 0.01 (n.s.)	0.04 \pm 0.04	0.04 \pm 0.03 (n.s.)
Bile	32.9 \pm 6.1	20.1 \pm 5.6 (p < 0.05)	53.2 \pm 3.5	45.0 \pm 4.5 (p < 0.05)
*(vol.)	(1.6 \pm 0.3)	(1.5 \pm 0.4) (n.s.)		
Liver	19.0 \pm 3.0	21.0 \pm 8.0 (n.s.)	31.7 \pm 1.3	38.2 \pm 3.3 (p < 0.05)
Rest of Tissues	9.3 \pm 1.5	8.2 \pm 1.7 (n.s.)	15.7 \pm 3.4	17.2 \pm 1.7

Figure 3.6

Distribution of ^{54}Mn in high speed liver supernatant fractionated on Sephadex G-75. (—), no pretreatment before iv injection of 1 mg/kg Mn; (---), pretreated with Zn ip (10 mg/kg) 24 hr before Mn administration. For purposes of comparison, the binding profile of ^{109}Cd in the liver cytosol of Zn-pretreated rats is also shown (——). Unlike Cd, Mn was not associated with the Mt fraction after pretreatment with Zn.

Table 3.3

The Excretion of [^3H]-L-dopa and Metabolites

The excretion of [^3H] three hours after the iv injection of L-dopa alone (4.1 mg/kg + 10 μCi [^3H]-L-dopa) or an equimolar solution of L-dopa + Mn (4.1 mg/kg L-dopa + 10 μCi [^3H]-L-dopa + 1.2 mg/kg Mn). Values are means \pm S.D. of four rats/group.

(n.s.) = not significant. Percentages were normalized by the arcsin transformation before comparing the means by Student's t-test.

	% of Total Counts Excreted	
	L-dopa alone	L-dopa + Mn
Urine	90.0 \pm 3.5	84.7 \pm 6.4 (n.s.)
Bile	10.0 \pm 3.5	15.3 \pm 6.4 (n.s.)

FIGURES

Figure 3.1

The typical time-course of biliary excretion of ^{54}Mn in individual rats in response to 3 different doses of the metal.

(—) 0.1 mg Mn/kg; (---) 1.0 mg Mn/kg;

(- - - -) 2.0 mg/kg Mn.

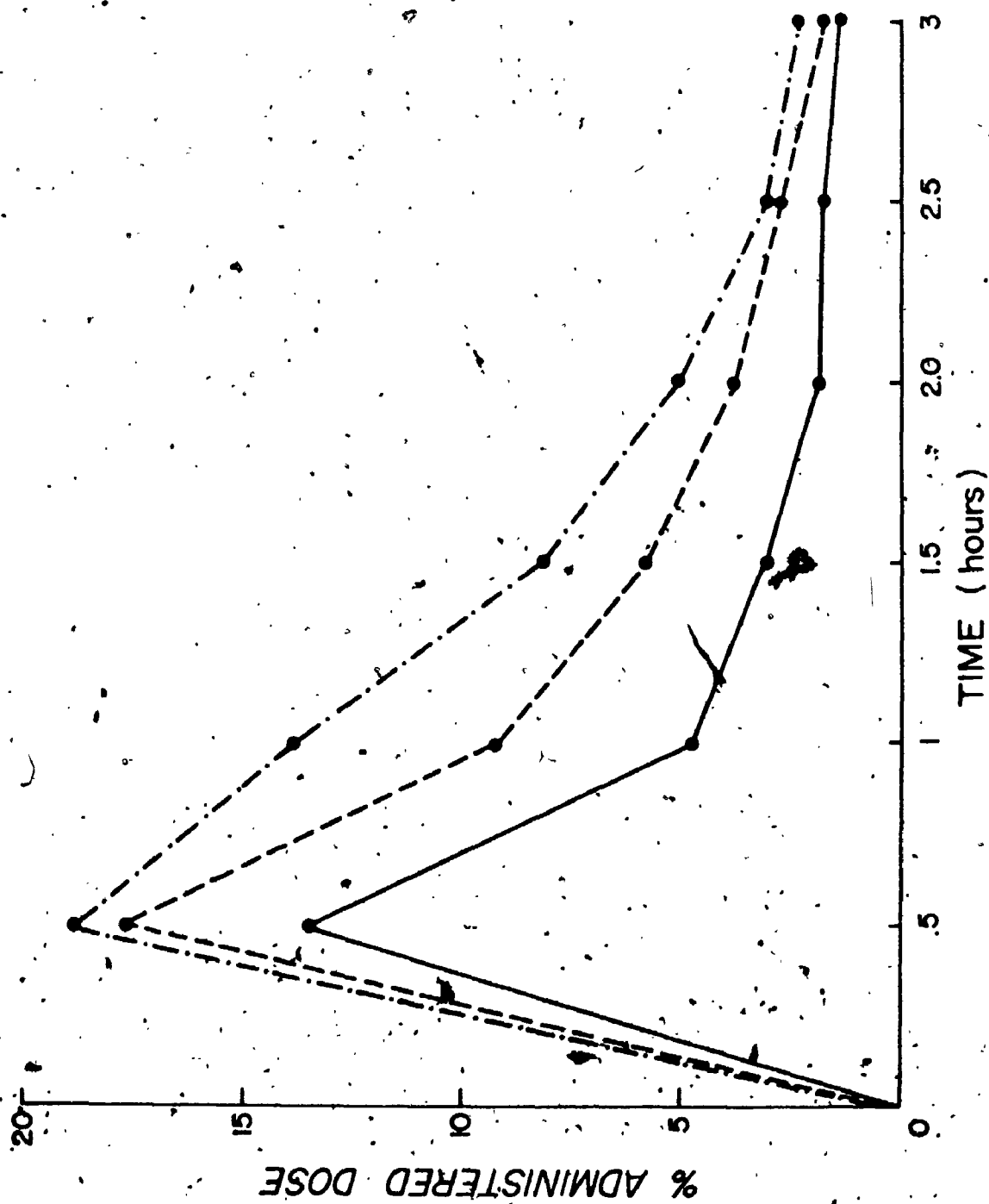


Figure 3.2

Differences among bile, liver, and other tissues with respect to Mn content 3 hr after the iv injection of 0.1, 1.0, or 2.0 mg Mn/kg.

Figure 3.3

The effects of increasing iv doses of Mn on the biliary excretion and tissue distribution of Mn. As the dose was increased, the % administered dose secreted into the bile increased, that remaining in the liver decreased slightly, and that taken up by kidney, pancreas, heart, as well as other tissues not shown, remained relatively unchanged 3 hr post-injection.

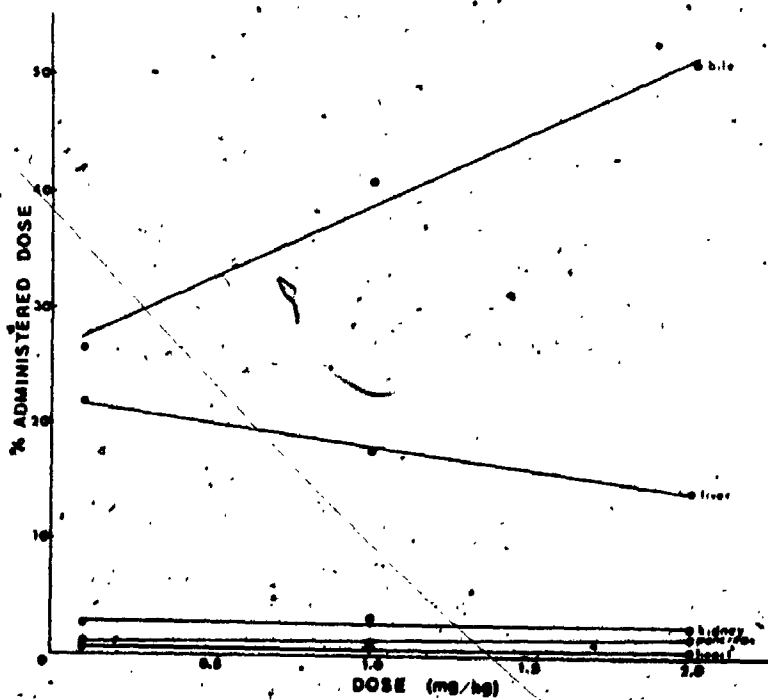
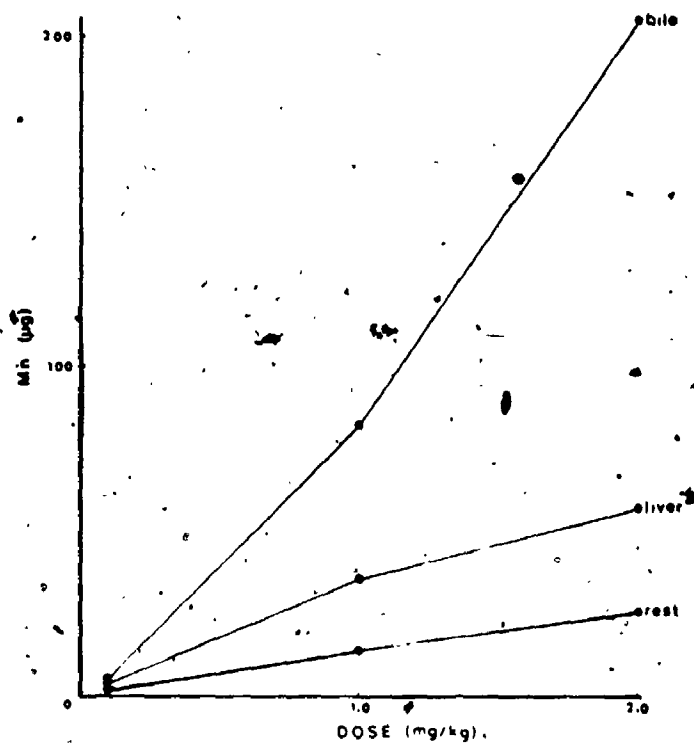


Figure 3.4

The binding of ^{54}Mn in rat bile as revealed by Sephadex G-75 chromatography. (—), bile collected for 1/2 hr after an iv injection of 1.0 mg Mn/kg labelled with ^{54}Mn ; (---), control bile to which a ^{54}Mn labelled solution of MnCl_2 was added in vitro.

Figure 3.5

The effect of 24 hr pretreatment with Zn (10 mg Zn/kg) on the biliary excretion of Mn (1.0 mg Mn/kg labelled with ^{54}Mn). (■), no pretreatment; (□), pretreated with Zn (n = 2). No obvious changes in the normal excretion pattern were noted.

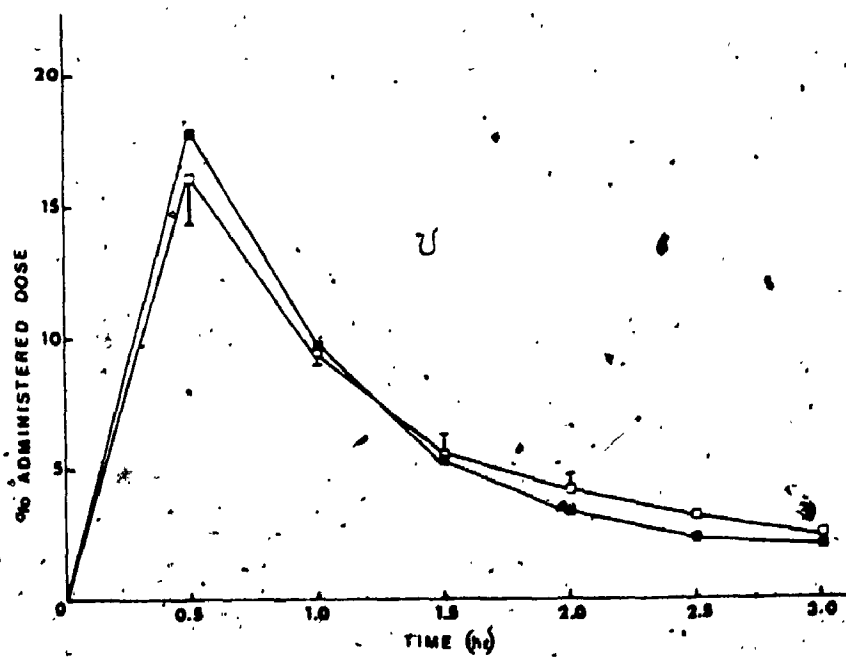
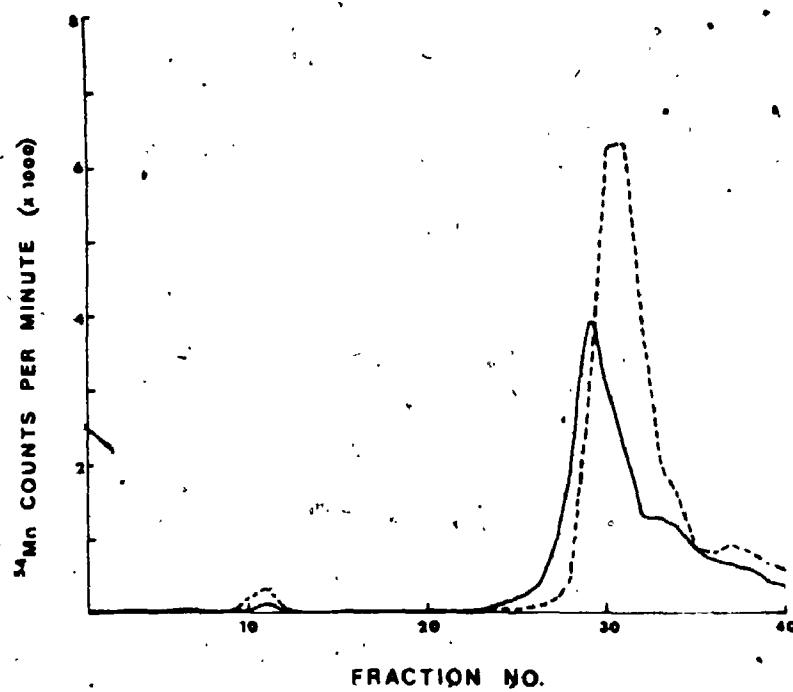


Figure 3.6

Distribution of ^{54}Mn in high speed liver supernatant fractionated on Sephadex G-75. (—), no pretreatment before iv injection of 1 mg/kg Mn; (---), pretreated with Zn ip (10 mg/kg) 24 hr before Mn administration. For purposes of comparison, the binding profile of ^{109}Cd in the liver cytosol of Zn-pretreated rats is also shown (——). Unlike Cd, Mn was not associated with the Mt fraction after pretreatment with Zn.

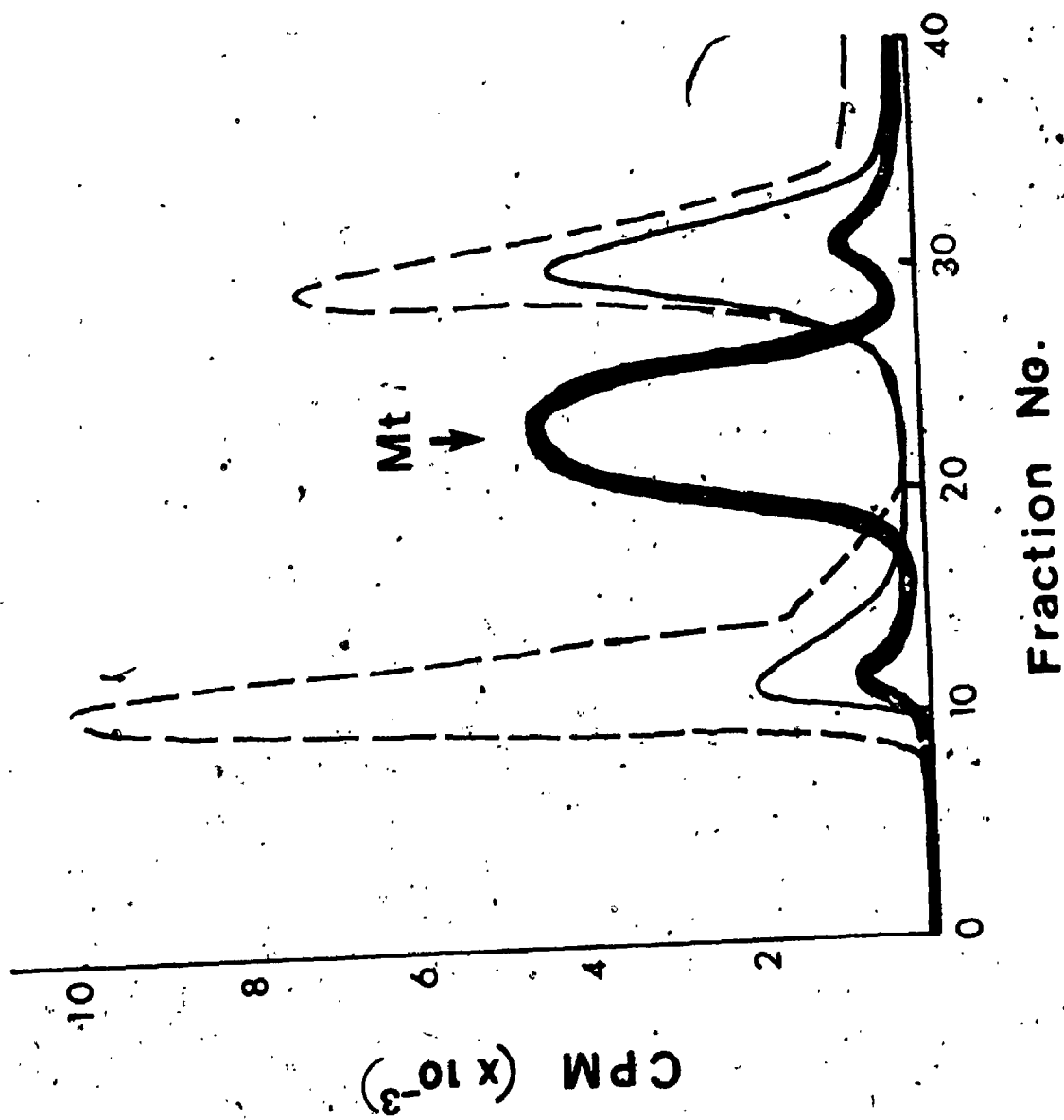


Figure 3.7

The time course of biliary excretion of Mn:

(■), Mn alone; (□), Mn + L-dopa. Also shown are the computer generated best fit exponential regression lines: (—), Mn alone, $r = -0.93$, $p < 0.01$; (---), Mn + L-dopa, $r = -0.84$, $p < 0.01$.

* = significantly different, $p < 0.05$

** = significantly different, $p < 0.01$

Figure 3.8

The effects of Mn on the biliary excretion of [^3H]-L-dopa and its metabolites. (■), L-dopa alone; (□), L-dopa + Mn. Inset: The corresponding best fit linear regression lines for the biliary excretion of [^3H]-L-dopa.

(—), L-dopa alone; (---), L-dopa + Mn.

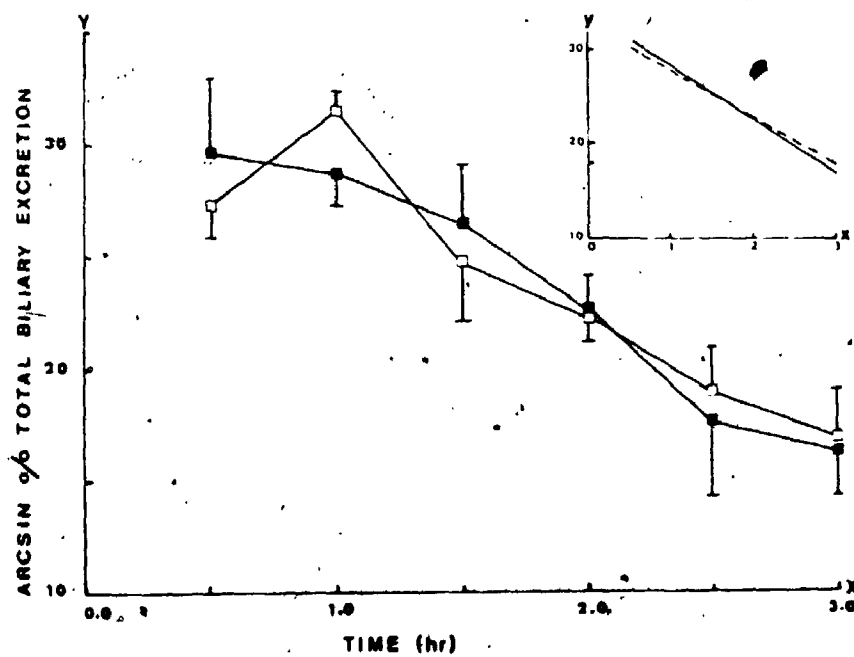
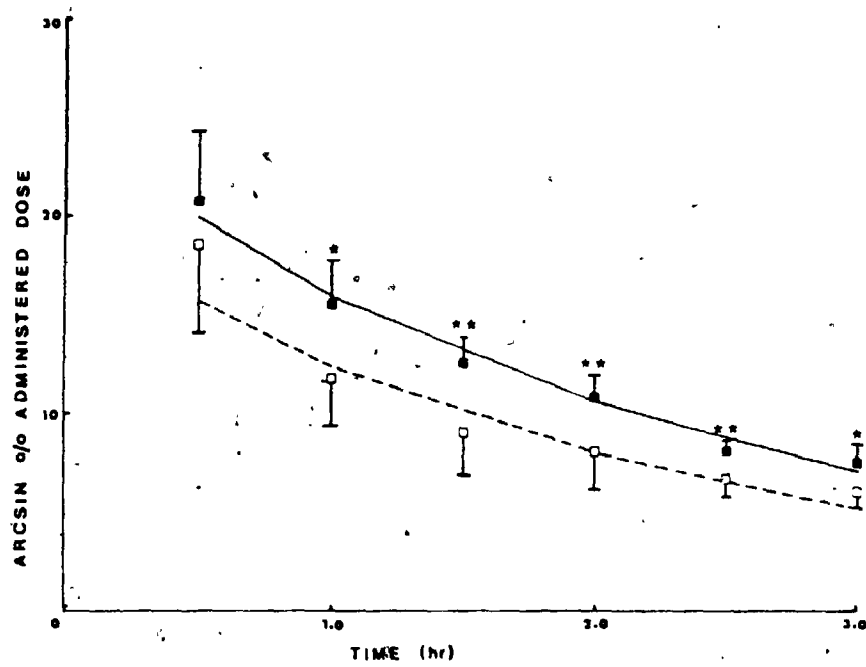


Figure 3.9

Sephadex G-75 elution profiles (buffer = 10 mM HEPES, pH 7.4) for the binding of ^{65}Zn , ^{109}Cd , ^{54}Mn , and ^{59}Fe to 5 mg purified rat albumin.

Incubations were for 1/2 hr at room temperature in 10 mM HEPES, pH 7.4. The binding of ^{54}Mn to albumin was not improved by changing the incubating and eluting buffer to 10 mM Tris, pH 8.6.

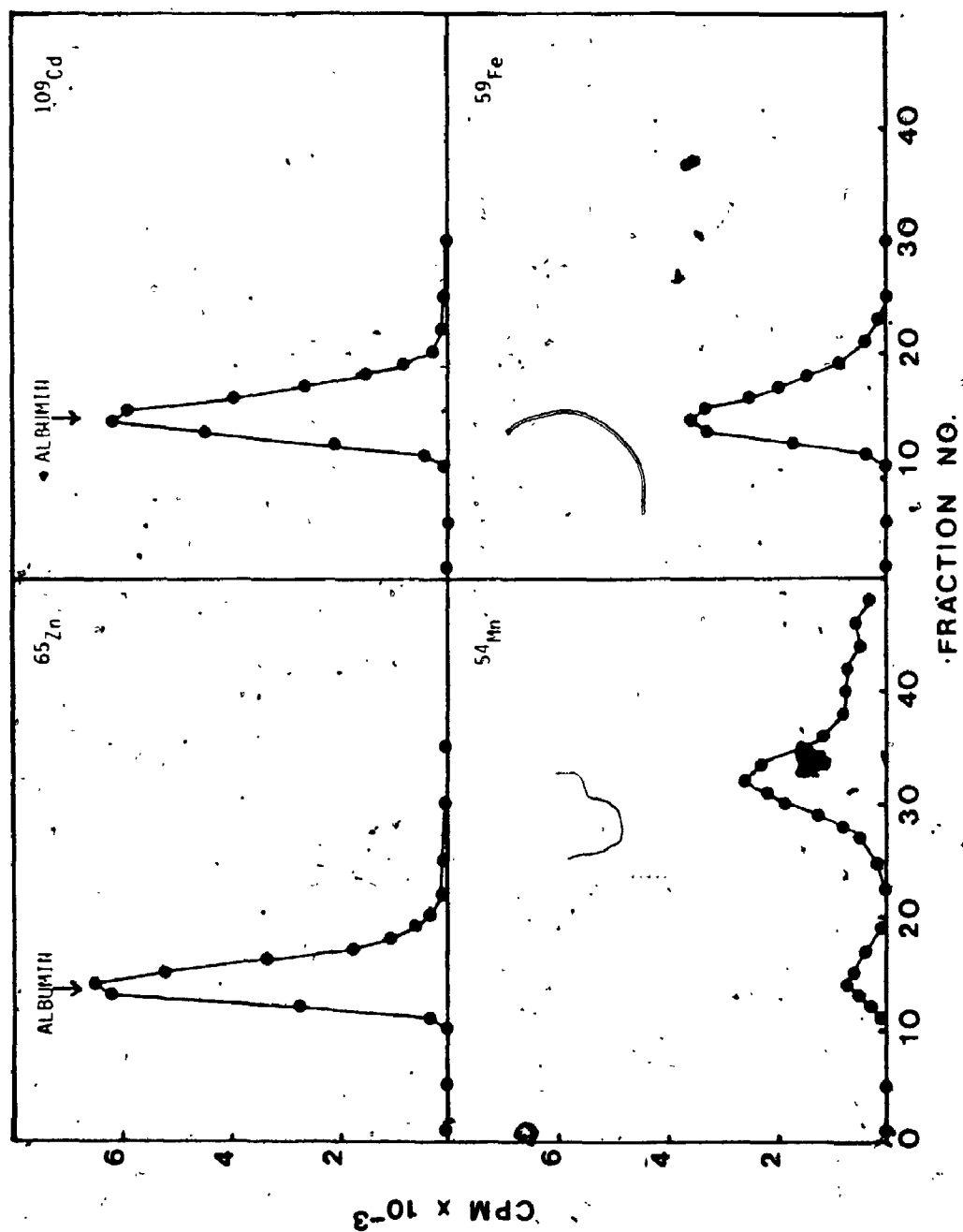
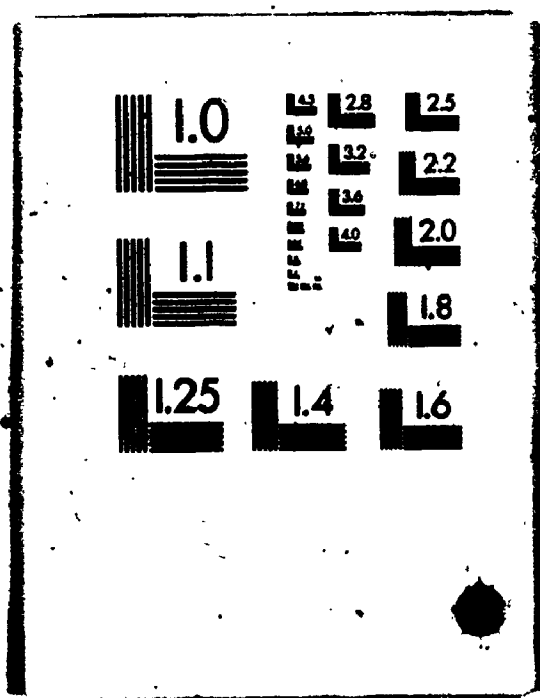


Figure 3.10

Elution profiles of ^{65}Zn radioactivity and ^{55}Mn content of fractions, from Sephadex G-75 fractionation of 5 mg albumin preincubated in the presence of $0.2\ \mu\text{Ci}\ ^{65}\text{Zn}$ for $1/2\ \text{hr}$, then $1\ \text{mM}\ \text{Mn}^{2+}$ for an additional $1/2\ \text{hr}$. Mn was unable to displace ^{65}Zn from the protein. Elution buffer = $10\ \text{mM}\ \text{HEPES}$, pH 7.4.

2



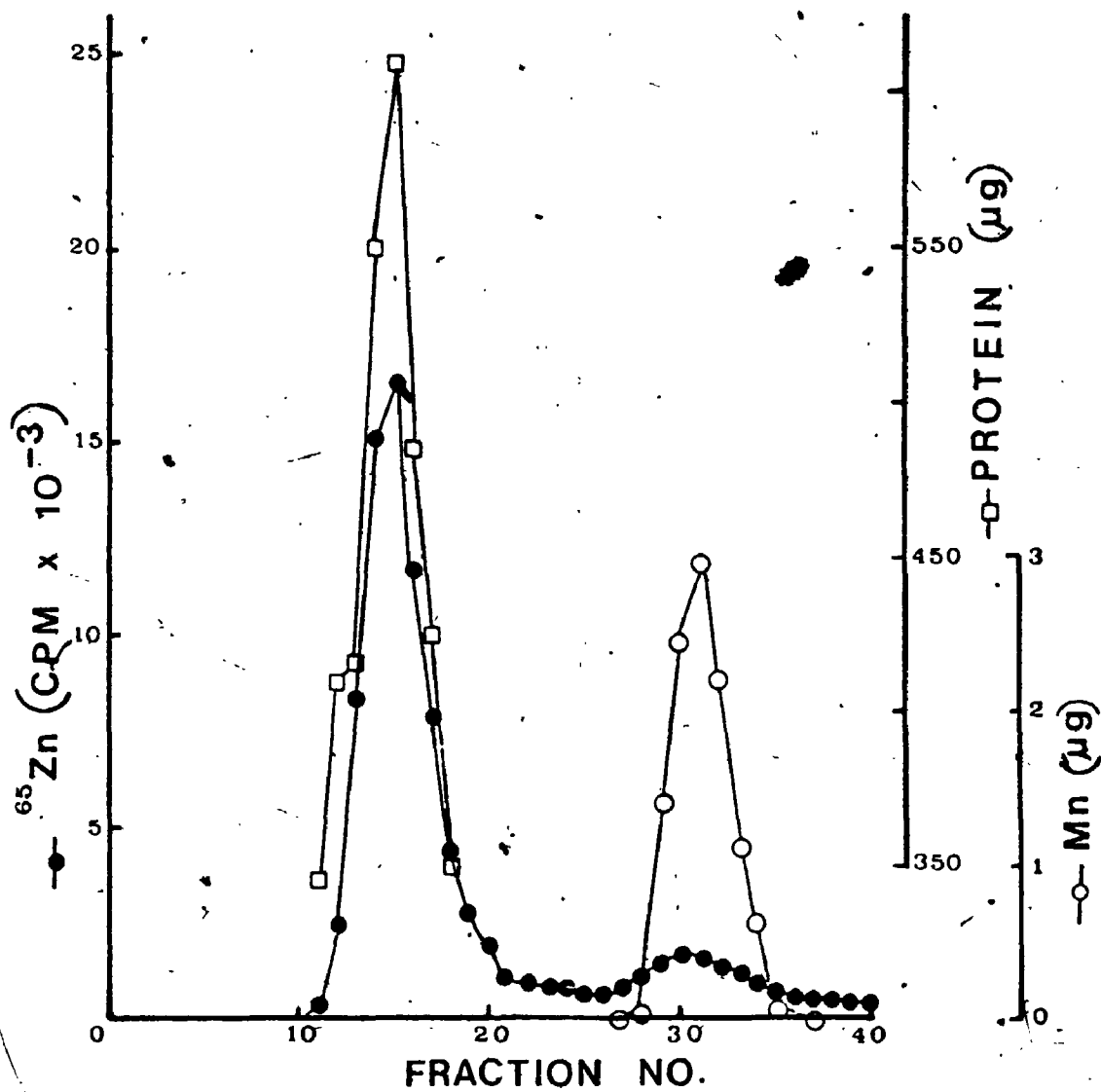


Figure 3.11

Sephadex G-75 elution profiles for the binding of ^{65}Zn , ^{109}Cd , ^{54}Mn , and ^{59}Fe to 5 mg purified human transferrin. Incubations were for 1/2 hr at room temperature in 10 mM HEPES, pH 7.4. The binding of ^{54}Mn to transferrin was improved by changing the incubating and eluting buffer to 10 mM Tris, pH 8.6,

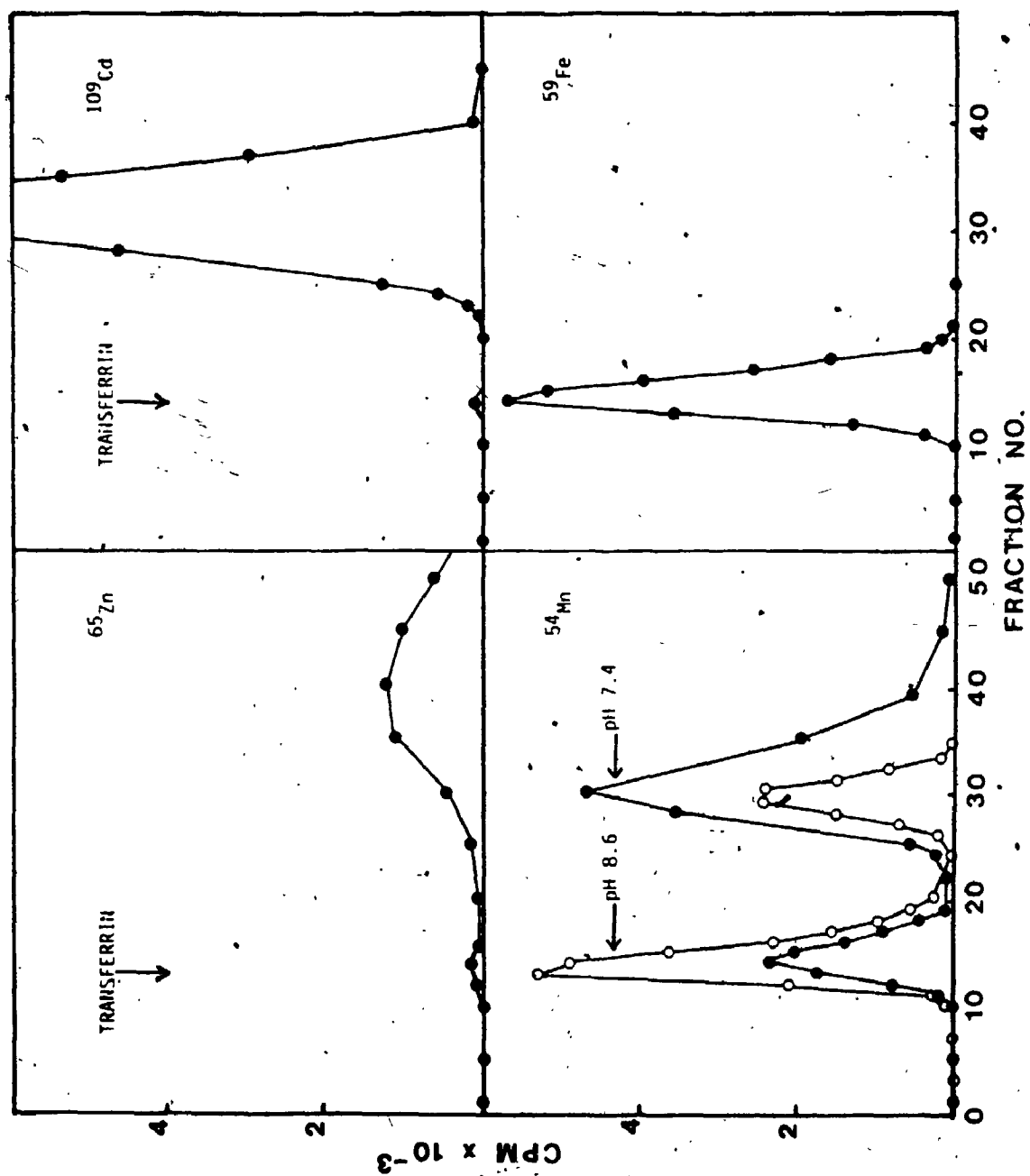


Figure 3.12

Sephadex G-75 elution profiles illustrating the prevention of ^{54}Mn binding to 5 mg purified transferrin by 1/2 hr preincubation in the presence of 7.8 μg Fe. a) without preincubation; b) preincubation with Fe.

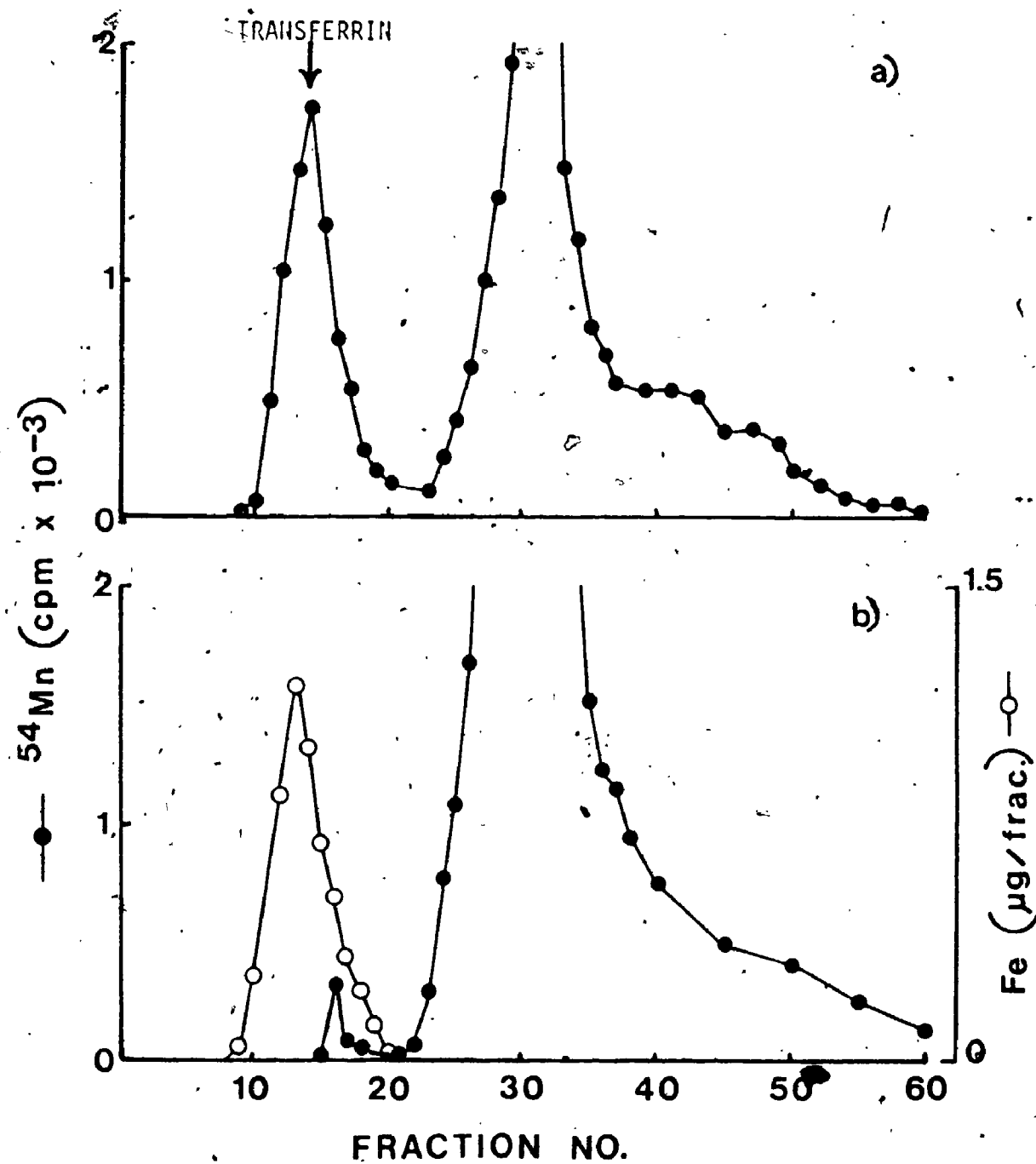


Figure 3.13

Sephadex G-75 elution profiles of 200 μ l human or rat plasma incubated with 0.14 μ Ci ^{54}Mn for 1/2 hr at room temperature in Tris buffer, pH 6.0 or 8.6 showing the effect of preincubation in the presence of 60 μ g ^{55}Mn on the binding of ^{54}Mn . (\bullet), no ^{55}Mn ; (\circ), 60 μ g ^{55}Mn ; (\square), ^{55}Mn content per fraction as measured by AAS.

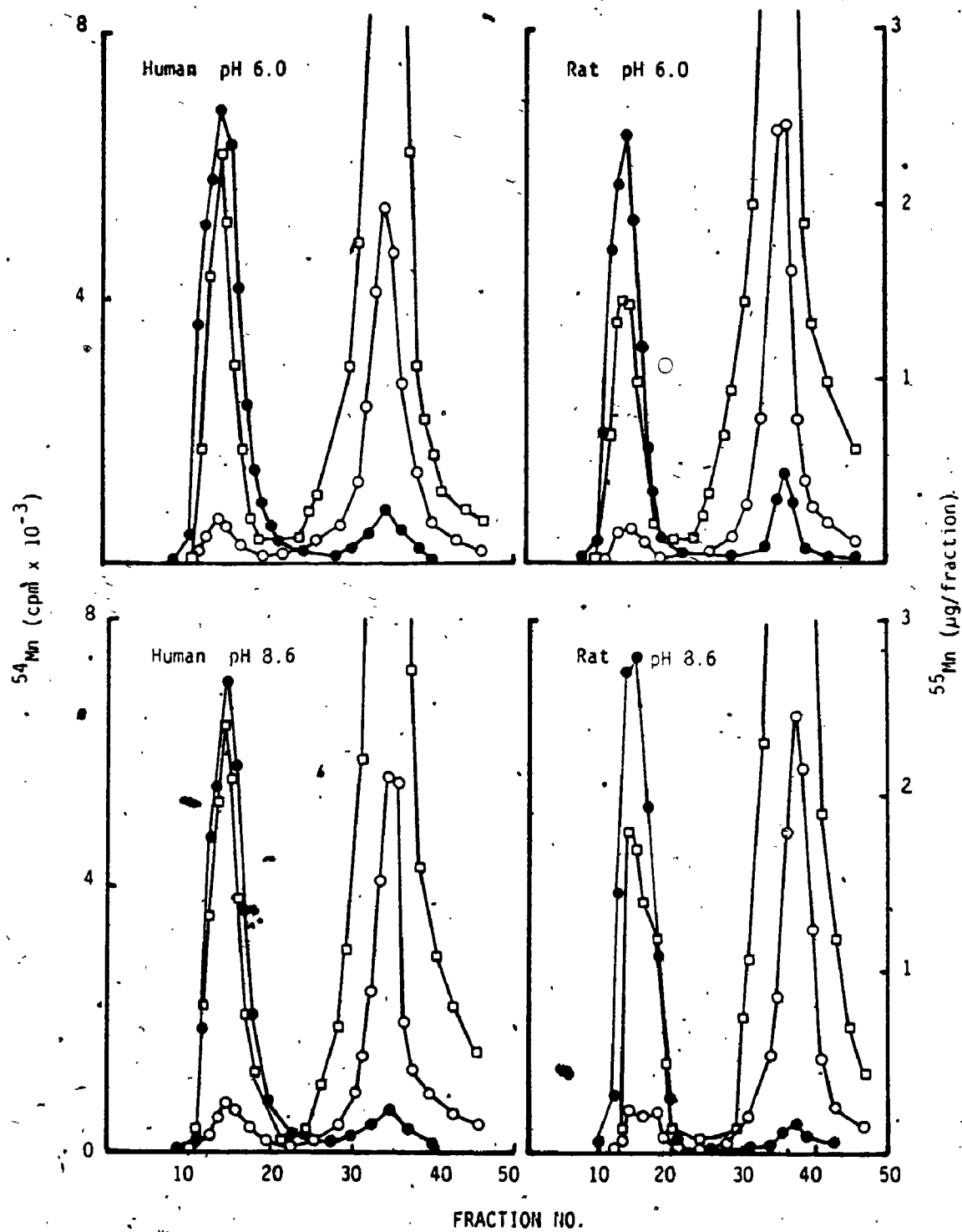


Figure 3.14

Sephadex G-75 elution profiles of 200 μ l human plasma incubated with 0.14 μ Ci ^{54}Mn for 1/2 hr at room temperature in Tris buffer, pH 6.0 or 8.6 showing the effect of preincubation in the presence of saturating amounts of Fe, Zn, and Cu on the subsequent binding of ^{54}Mn to HMW proteins. Preincubation of plasma with Fe, Zn, and Cu had little effect on ^{54}Mn binding to plasma proteins.

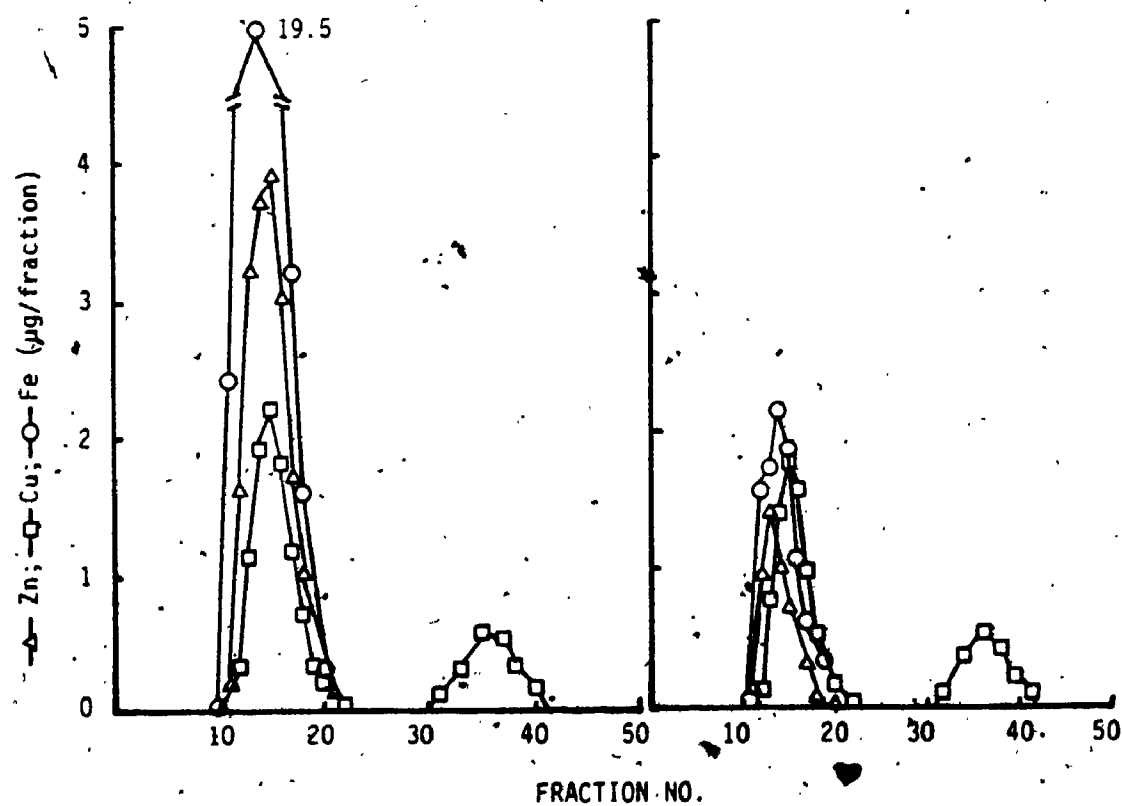
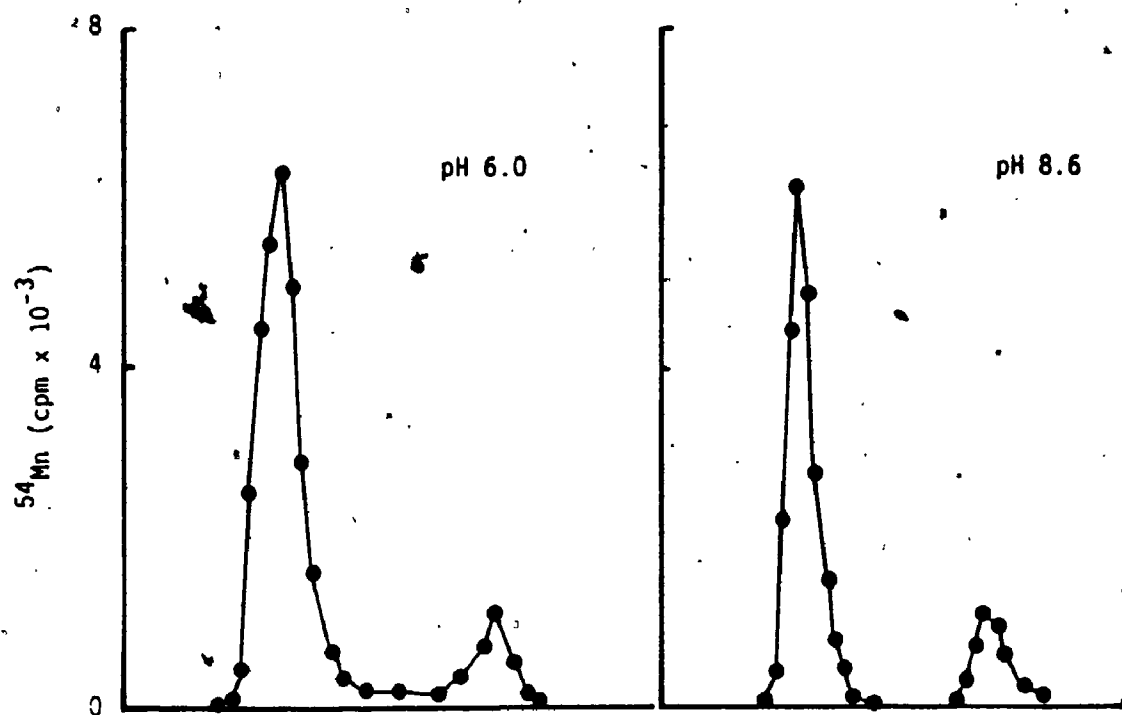
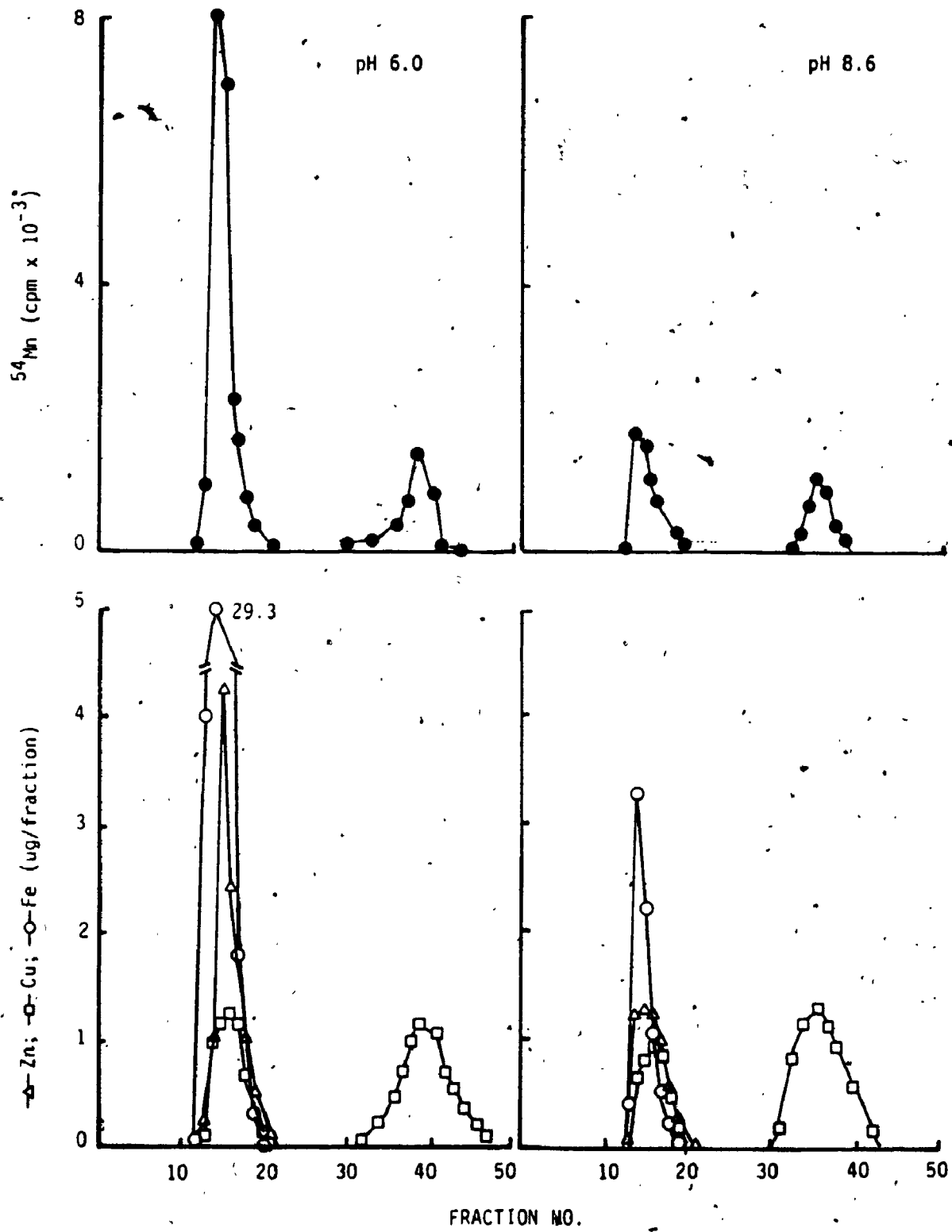


Figure 3.15

Sephadex G-75 elution profiles of 200 μ l rat plasma incubated with 0.14 μ Ci 54 Mn for 1/2 hr at room temperature in Tris buffer, pH 6.0 or 8.6 showing the effect of preincubation in the presence of saturating amounts of Fe, Zn, and Cu on the subsequent binding of 54 Mn to HMW proteins. Preincubation of plasma with Fe, Zn, and Cu caused a significant decrease in the binding of 54 Mn to plasma proteins at pH 8.6 but not at pH 6.0.



CHAPTER FOUR

THE ACCUMULATION AND EFFECTS OF SUBCHRONICALLY INJECTED Mn

4.1 Methods

4.1.1 Animals and Treatment

Male Sprague-Dawley rats (100-200 g) obtained from Canadian Breeding Farm and Laboratory, St. Constant, Canada, were divided into 2 groups. Group 1 received daily ip injections of 3.0 mg Mn/kg body weight as $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ or $\text{Mn}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 4\text{H}_2\text{O}$ for 30 days. Group 2 served as the control and received equal volumes of 0.9% NaCl. Both groups had free access to standard rat chow (Purina Co.) and tap water ad libitum. At the end of the experimental period, an additional 48 hr span was allowed for the excretion of any unbound Mn, then all animals were sacrificed under pentobarbital anesthesia by aortal exsanguination with a heparinized syringe. Blood, brain, heart, lungs, kidneys, liver, spleen, pancreas, duodenum, testes, skeletal muscle, and bone were removed for analysis.

4.1.2 Regional Dissection of Rat Brain

To isolate discrete brain regions, dissections were performed in a cold room maintained at 4°C. Individual brains were placed on the clean outer surface of a culture dish (Pyrex, 150 mm diameter) with crushed

ice beneath the glass. This procedure is recommended since brain tissue which is not kept at near freezing temperatures loses its firmness and becomes virtually impossible to work with.

Two basic tools were used to perform the dissections: a No. 11 scalpel blade for cutting, and a set of small curved serrated forceps for holding and pinching off tissue. Also essential is some source of magnification such as a dissection microscope. For the work presented here, an illuminated magnifier with a circumferential fluorescent lamp was employed.

First, the pons-medulla and cerebellum were separated from the remainder of the brain. A transverse section was made, as shown in Fig. 4.2, at the junction of the midbrain and pons. The cerebellum was then removed from the pons-medulla by severing the peduncles.

The olfactory bulbs were separated from the cortex by pinching off the bulbs with forceps.

A vertical section through the brain at the level of the optic chiasm was performed (level "A", Figs. 4.1 and 4.2). This corresponds to section A22 from Craigie (1963) and A7470 from Konig and Klippel (1963). This cut allows access to the corpus striatum and hypothalamus, and also separates the "frontal cortex" from the "rest of the cortex".

The anterior portion was bisected by midline section and each of the resulting halves separated into cortex, corpus callosum, and corpus striatum. First, the striatum was removed by making a series of small scalpel cuts to separate the striatum from the corpus callosum and other adjacent tissue. Then forceps were used to pinch off and lift out the striatal tissue. The same procedure was later repeated to dissect the remaining mass of striatum from the posterior portion of the brain.

(Fig. 4.3). The corpus callosum was separated from the grey matter of the frontal cortex by peeling the white matter away with forceps while making shallow cuts with the scalpel to free the callosum from the cortical tissue.

The posterior portion of the brain resulting from section "A" was divided into rest of cortex, corpus callosum, hippocampi, thalami, hypothalamus, colliculi, tegmentum, amygdalae and corpus striatum.

The amygdalae were isolated bilaterally by cutting the lateral surface of the hemispheres along with rhinal fissure to a point just posterior to the level of the mammillary bodies. A second, similar cut was made obliquely from a point lateral to the attachment of the optic chiasm (Figs. 4.1 and 4.2). The resulting block of tissue consisted primarily of the amygdaloid nuclei although a small amount of pyriform cortex was also present.

The tectum (inferior and superior colliculi) was dissected by approaching the brain posteriorly, parting the overlying flaps of cortex to expose the colliculi, and making cuts around the midbrain down to the level shown in Fig. 4.3. Tissue consisting primarily of the tectum was then pinched off and lifted out with forceps (see also Fig. 4.4 for relative position of colliculi).

The tegmentum was then isolated by cutting the remainder of the midbrain away from the rest of the brain (see Figs. 4.1 and 4.3 for guidelines).

The dissection of the hypothalamus was accomplished by cutting around this region, which is depicted in Fig. 4.1, using the anterior commissures (Fig. 4.2) as landmarks for the proper depth of the cut. The hypothalamic tissue was then lifted away with forceps and the overlying white matter of the optic chiasm was trimmed off.

The striatal tissue of the posterior portion of the brain was isolated in a similar manner to that described previously for the striatal tissue of the anterior portion. The corpus callosum and lateral ventricles (Fig. 4.2) served as landmarks for the dissection. See also Fig. 4.4 for an indication of the tapering shape of the corpus striatum. The septal nuclei, which were not included in the present dissection, lie between the left and right ventricles and could be removed at this point if desired.

Next, the thalami were readily freed from the overlying cortex, the hippocampi were peeled away from the closely adhering cortex using forceps, and the corpus callosum was separated from the cortex by the procedure previously described for the anterior portion of the brain.

Table 4.1 gives a list of the average dry weights obtained for the 13 discrete regions. These weights are accurately reproducible once the dissection technique has been mastered.

For HPLC analysis of dopamine (DA), homovanillic acid (HVA), 3,4-dihydroxy-phenyl-acetic acid (DOPAC), norepinephrine (NE), 5-hydroxytryptamine (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) in the corpus striatum, pons-medulla, and frontal cortex, brains were placed immediately on dry ice after sacrificing the animals and stored at -80°C until analysis. Dissections were performed over dry ice.

4.1.3 Metal Analysis

Whole organs in the case of heart, lung, kidney, spleen, pancreas, duodenum, and testis, or ~ 0.4 g of tissue in the case of liver, muscle and bone, were freeze-dried (Model 10-030 lyophilizer, Virtis Co., Inc., Gardiner, N.Y.), weighed, and digested in HNO_3 (reagent grade, Fisher Scientific Co., Toronto, Canada). Heating overnight in an oven maintained at 75°C facilitated digestion. The resulting clear yellow solutions were made up to 2.0 ml with distilled-deionized H_2O and analyzed for metal content by atomic absorption spectrophotometry using a Jarrell-Ash Model 810 equipped with dual-beam background correction and an FLA-10 flameless atomizer. The metals that were analyzed and the corresponding analytic wavelengths used are as follows: Mg, 2852 Å; Zn, 2139 Å; Fe, 2483 Å; Cu, 3247 Å; and Mn, 2795 Å. Mn and Cu were estimated by flameless technique, others by flame mode (air-acetylene flame). In the analysis, linear calibration curves were obtained over the following ranges of concentration: Mg, 0.1-2.0 ppm; Zn, 0.1-2.0 ppm; Fe, 0.5-5.0 ppm; Cu, flameless, 0.05-0.5 ppm; Mn, flameless, 0.01-0.20 ppm. Samples were analyzed in duplicate or triplicate.

A 1 ml aliquot of whole blood was taken for analysis of metals. The remaining volume of blood (~ 7-9 ml/rat) was centrifuged at $900\times g$ for 15 min. to separate plasma and cells. A 1 ml aliquot of plasma was taken for estimation of metal content, and the erythrocyte pellet was resuspended, washed with 0.9% NaCl and recentrifuged three times. A 1 ml aliquot of washed erythrocytes was taken for metal analysis. The remaining red cells were lysed with 10 volumes of distilled-deionized H_2O and the membrane stroma was removed by centrifugation. The resulting hemoglobin solution was dialyzed (Spectrapac membrane tubing,

A.H. Thomas Co., Philadelphia, PA) against a large volume of 0.005 M Tris, pH 8.6, in a cold room (4°C), then freeze-dried, weighed, and digested in HNO₃ prior to metal analysis.

4.1.4 Subcellular Fractionation

The subcellular distribution of Mn in the kidney was determined. The following fractions were obtained by the method of Sabbioni and Marafante (1976) from 20% kidney homogenates in 0.25 M sucrose-TKM buffer, pH 8.6, by differential centrifugation: crude nuclear (600x g, 10 min), crude mitochondrial (8,000 g, 10 min), lysosomal (30,000x g, 15 min), microsomal (105,000x g, 60 min), and cytosol (supernatant). Acid digested fractions were analyzed for Mn content by flameless atomic absorption spectrophotometry.

4.1.5 Histological Examination of the Pancreas

After sacrifice, pancreatic tissue was removed immediately and a portion not used for metal analysis was fixed in 10% buffered formalin for several days before embedding in paraffin. Sections of 5 µm thickness were cut and stained with H & E, Masson trichrome, and Prussian blue.

4.1.6 HPLC Analysis and Dopamine Receptor Estimation

The measurement of biogenic amines and their metabolites was accomplished by HPLC with electrochemical detection by modifications of the methods originally devised by Refshauge et al. (1974) and Keller et al. (1976).

Brain regions were homogenized in 1.0 ml of 0.1 M HClO_4 /1 mM EDTA (frontal cortex, pons-medulla) or 1.0 ml of 15 mM Tris/1 mM EDTA, pH 7.4 (corpus striatum); 150 μl of the homogenate was taken for metal analysis, and the remainder was centrifuged at 20,000x g for 10 min. 500 μl of the supernatant was taken for separation of catechols by adsorption on alumina. The remainder was acidified (necessary for striatal tissue only) with 10 μl of 1.0 M HClO_4 /10 mM EDTA and frozen until HPLC analysis for 5-HT, 5-HIAA, and HVA. The pellet was discarded except in the case of striatal tissue in which case the pellet was resuspended in 15 mM Tris/5 mM EDTA and ^3H -SPIRO binding assays modified from Seeman et al. (1976) were performed to estimate DA-receptor density (see section 7.1.2 for details on binding assay).

Adsorption/desorption of catechols (DA, NE, DOPAC) on alumina was carried out as follows: 500 μl aliquots of supernatant were added to plastic 1.5 ml Eppendorf tubes containing ~ 50 mg acid washed alumina. The pH was adjusted to 8.6 by the addition of 0.5 M Tris, pH 9.0. Tubes were capped and agitated for 5 min, then spun for 1 min in an Eppendorf 5414 centrifuge. The supernatant was discarded, the alumina was washed once with 1.0 ml distilled-deionized H_2O , and catechols were desorbed by 500 μl of 0.1 M HClO_4 /1 mM EDTA.

The HPLC system utilized a glassy carbon electrochemical detector and power supply from BAS, Inc., and a C-18 reverse phase column and Model 510 pump from Waters Co. The running buffer (mobile phase) for the determination of DA, NE, and DOPAC was 0.07 M phosphate/1.0 mM EDTA/0.50 mM sodium octyl sulphate, pH 3.8, containing 8% methanol. For 5-HT, 5-HIAA, and HVA estimations, the methanol content was increased to 16%.

Appropriate concentrations of standards (NE, DA, DOPAC, HVA, 5-HT, 5-HIAA from Sigma) were processed in an identical manner to tissue samples with regards to homogenization, centrifugation, adsorption/desorption, etc. Epinine (deoxyepinephrine) was used as the internal standard. Typical chromatographs are depicted in Figs. 4.14 and 4.15.

4.2 Results

4.2.1 Blood Metal Content

Blood Mn levels for control and Mn treated rats are given in Table 4.2. The Mn concentration in whole blood of control rats was too low to be estimated accurately by AAS, but levels were higher in erythrocytes than in plasma in accord with previous reports for human and rabbit blood (Papavasiliou and Cotzias, 1961). Similarly, the increase in whole blood Mn content in the Mn treated group was almost totally accounted for by the increase in the Mn content of the erythrocyte fraction. The increase in plasma Mn seen in the treated group, although

significant, represented a negligible fraction of the total increase in blood Mn. Furthermore, the dialyzed (14,000 molecular weight cut-off), dried hemolysate of Mn treated rats had an Mn concentration approximately twice that of whole dry erythrocytes indicating that stromal, membrane-bound Mn did not contribute in a major way to the observed increase in red cell Mn content.

Table 4.3 depicts the effects of chronic Mn administration on the levels of Zn, Fe, and Cu in erythrocytes and plasma. No significant changes from the normal condition were noted in the red cells, but plasma Zn decreased and plasma Cu increased following Mn treatment.

4.2.2 Tissue Metal Content

A comparison of Mn concentrations within 13 brain regions of control and treated rats is given in Fig. 4.5. Highest normal concentrations of the metal were in hypothalamus, colliculi, olfactory bulbs, and midbrain. Due to treatment, all brain regions showed significant increases in Mn concentration with an average increase of about 49 nmol Mn/g dry tissue. The greatest increase was 135 nmol Mn/g in the corpus striatum followed by the thalamic area and the midbrain with increases of 77 and 67 nmol Mn/g, respectively. The corpus callosum, cerebellum, and amygdala also had higher than average accumulation. The corpus callosum had the highest percentage increase (1300%).

Table 4.4 summarizes the differences in distribution of Zn, Cu, and Fe between the control and treated groups.

Zn was highest in the amygdala, cortex, hippocampus, and olfactory bulb of the control group. There was a significant decrease in the amygdala and hypothalamus after treatment.

The hypothalamus, cerebellum, striatum, and hippocampus showed the highest normal concentrations of Cu. Cu increased in several brain regions due to Mn treatment.

There were no significant changes in Fe distribution as a consequence of Mn administration. Levels of Fe were normally highest in the hypothalamus, colliculi, olfactory bulb, and striatum.

As a general statement, areas rich in white matter, such as the corpus callosum and brainstem usually had the lowest metal concentrations under normal conditions and areas rich in gray matter, such as hypothalamus, olfactory bulb, and cortex had higher heavy metal concentrations.

Attempts were made to correlate the distribution of Mn in brains of normal rats with the distribution patterns of the other heavy metals examined. Mn distribution correlated well with that of Fe (Fig. 4.6), and Cu (Fig. 4.7), but not with Zn (Fig. 4.8).

Table 4.5 summarizes the Mn, Mg, Zn, Fe and Cu concentrations in tissues other than brain of control and Mn treated rats. The highest tissue concentration of Mn in control rats was found in the pancreas followed closely by liver. Skeletal muscle revealed the lowest control tissue Mn levels. The tissue with the greatest increase in Mn content due to treatment was bone in which Mn concentration rose by approximately 37 $\mu\text{g/g}$. In the soft tissues, Mn content increased

maximally in the pancreas and the increase was almost as high as that in bone, ~ 32 µg/g above control. This amount was far greater than that seen in any of the other soft tissues, all of which revealed increases of 10 µg/g (duodenum) or less. Typically, organs exhibited a three- to four-fold increase in Mn concentration over their respective control levels due to treatment (Table 4.6). Liver was atypical in this regard, exhibiting little or no increase in Mn concentration due to treatment. On the other hand, three of the soft organs - pancreas, duodenum and testes - showed approximately a six-fold elevation in Mn content. Thus, the pancreas revealed not only the highest absolute increase in Mn concentration but also one of the highest percentage increases.

Mn treatment had little effect on the normal tissue distribution of Mg and Zn (Table 4.5). Bone, however, showed a 10% decrease in the concentrations of both Mg and Zn. Fe was found to be increased by almost 200% in pancreatic tissue of Mn treated rats. However, the most widespread effect of chronic Mn treatment on the distribution of other metals was a significant increase in the Cu content of several tissues, especially those having relatively low control Cu levels. As already noted, plasma Cu levels were also increased in the Mn-exposed group.

4.2.3 Subcellular Mn Distribution

The subcellular distribution of Mn in renal tissue of control and Mn treated rats is summarized in Figure 4.9. The crude nuclear fraction contained the highest proportion of Mn in both groups. The concentration of Mn increased in all fractions due to treatment. The crude nuclear fraction exhibited the greatest absolute increase ($\sim 1.5 \mu\text{g/g}$) whereas the mitochondrial fraction revealed the greatest percentage increase ($\sim 600\%$). The pattern of subcellular Mn distribution was similar in both the control and treated groups, thus, there was no strong tendency towards a selective accumulation of Mn in any particular subcellular fraction.

4.2.4 Histological Appearance of Pancreatic Tissue

Figure 4.10, a and b, show the histological appearance of pancreatic tissue from control rats. In control animals injected only with 0.9% NaCl, both endocrine (islets of Langerhans) and exocrine (acini and ducts) portions of the pancreas appeared morphologically normal. Extracellular spaces were narrow and the gland was covered by a thin connective tissue capsule. The presence of connective tissue other than the delicate outer capsule and interlobular septa, was negligible. Large portions of pancreatic tissue from Mn treated rats also appeared normal, but frequently areas were seen that exhibited pathological changes (Figs. 4.11, 4.12, 4.13). These changes included: 1) expanded interacinar spaces indicating the presence of edema fluid; 2) dilated

blood vessels; 3) a much thickened connective tissue capsule with fibrotic invaginations extending into the body of the gland; 4) the presence in this connective tissue of fibroblasts and, frequently, inflammatory cells including neutrophils, lymphocytes, and macrophages; 5) the separation of small groups of acini from the body of the pancreas and occasional destruction of acini. Staining for the presence of iron revealed an increased amount of stainable iron in pancreatic tissue from Mn-exposed rats. This iron was localized in the proliferating connective tissue, and was never seen within islets or secretory acini. Masson trichrome staining confirmed the presence of a fine network of collagen fibres within the new connective tissue indicative of fibrosis. Other organs in the peritoneal cavity, such as liver and spleen did not exhibit significant pathological changes, nor did they accumulate high concentrations of Mn.

4.2.5 DA, HVA, DOPAC, NE, 5-HT and 5-HIAA Concentrations

In response to the daily ip injection of 3.0 mg Mn/kg for 30 days, the DA content of the frontal cortex and pons-medulla, and the NE content of the pons-medulla were found to be elevated compared to control (Tables 4.7 and 4.8), whereas in the corpus striatum, no changes in the concentrations of any of the measured neurotransmitters or their metabolites were noted (Table 4.9), even though the accumulation of Mn was higher in the striatum. The density of striatal D2 DA-receptors was

likewise unaltered due to Mn treatment (Table 4.9). Central serotonergic parameters (5-HT, 5-HIAA) were unchanged due to Mn treatment.

4.3 Discussion

The mechanism by which Mn exerts its toxic effects on the CNS is not understood and little is known concerning its uptake and distribution in the CNS. Although postmortem examinations have been performed on brains of patients who suffered from manganism (Canavan et al., 1934; Bernheimer et al., 1973), measurements of metal concentration have not been done. There is a similar lack of regional data for brains of animals experimentally exposed to Mn. In such experiments Mn estimations have either not been performed (Neff et al., 1969; Pentschew et al., 1963) or, if they have, only whole brain Mn concentrations have been reported (Chandra and Srivastava, 1970; Shukla and Chandra, 1979; Chandra et al., 1980). Yet it has been known for some time that regional differences in the distribution of trace metals exist in both animal (Donaldson et al., 1973; Haug, 1973; Fjerdingsstad et al., 1974) and human (Cumings, 1968; Harrison et al., 1968) brain.

Pentschew et al. (1963) studied primates chronically exposed to Mn and demonstrated severe selective damage to two components of the extrapyramidal system, the medial pallidum, and the subthalamic nuclei. Similar lesions of the corpus striatum, as well as damage to the substantia nigra (midbrain) and thalamus have been observed in brains of

workers who suffered from the neurological syndrome typical of Mn poisoning (Barbeau et al., 1976). The present study has demonstrated that under conditions of chronic exposure, Mn is accumulated by striatal, midbrain, and thalamic regions at a greater rate than many other brain areas. These data support the contention that the neuronal damage inflicted by Mn occurs as a result of the metal being selectively concentrated by, and thus more quickly reaching toxic levels in, certain nuclei of the extrapyramidal system. Other brain regions also take up Mn, but at slower and varying rates. This may partially explain the variety of secondary signs and symptoms occasionally reported in cases of manganism such as psychotic behavior, memory loss, speech difficulty, loss of libido, and hallucinations (Mena et al., 1967). The primary target, however, as evidenced by the relatively rapid accumulation of Mn, seems to be extrapyramidal structures.

The significant decrease in Zn concentration in amygdala and hypothalamus due to Mn administration may correlate with the findings of Singh et al. (1974) who noted a 41% decrease in acid phosphatase and a 24% decrease in alkaline phosphatase activity in rat brain after chronic Mn exposure. These are both Zn-containing enzymes. Certainly, Zn loss in general has been associated with impaired mental function. Brain autopsy specimens from schizophrenics contained approximately half as much Zn as brains of patients with various other kinds of neural disorders (Kimura and Kimura, 1965). Also, Henkin et al. (1975) reported psychotic episodes, paranoid delusions, depression, and other changes in mental state following acute Zn loss due to L-histidine administration in humans. These symptoms were subsequently alleviated following ZnSO_4 therapy. Whether there may be a connection between

decreased Zn and the so-called "manganese psychosis", which has been well documented in miners suffering from Mn poisoning (Mena et al., 1967), requires further investigation.

Increases in whole-brain Cu concentrations following chronic exposure to Mn have been reported (Gubler et al., 1954; Singh et al., 1979). The results of the present study confirm and extend these findings. Mn treatment caused increases in Cu in several brain regions with the greatest increases associated with corpus callosum, thalamus, and cortex; however, it may be that increases in brain Cu are nonspecific and may simply be due to the increased plasma Cu levels also observed during experimental Mn poisoning (Table 4.3).

Under the present experimental conditions, no change in Fe levels due to Mn treatment were observed in any brain region examined. However, it is interesting to note the highly significant positive correlation between Mn and Fe distribution in brains of untreated rats (Fig. 4.6). Regions which had a high Mn level also had a high Fe concentration. A similar but less striking relationship was noted between Mn and Cu (Fig. 4.7). Conversely, no correlation was observed between Mn and Zn distributions (Fig. 4.8). The existence of a physiological relationship between Mn and Fe is supported by the findings of others: (1) there is direct competition between Fe and Mn in gastrointestinal absorption (Thomson et al., 1971); (2) prolonged exposure to excess Mn causes anemia (Gubler et al., 1954); (3) uptake of Mn by brain is swifter in anemic rats (Mena, 1974). In the human brain, the pallidum has a higher level of Fe than other nuclei (Hallgren and Sourander, 1958; Cumings, 1968) and it is this structure that is selectively damaged in cases of manganese intoxication in primates.

Pentschew et al. (1963). It is thus possible that one of the mechanisms by which Mn exerts its toxic effect is by the displacement of Fe or interference with other essential metals in this brain region. Evidence is accumulating that suggests that nonessential metals, such as Hg, Pb, and Cd, exert toxic effects by interacting with essential metals, thereby adversely affecting various metabolic processes (Finelli et al., 1975; Goyer, 1978; Petering, 1978; Prohaska and Ganther, 1977). It is reasonable to speculate that long-term exposure to abnormally high levels of Mn might result in consequences of a similar nature. However, in the present study, striatal Fe was not significantly altered after Mn treatment indicating that a longer treatment time or higher doses of Mn may be required to show a statistically significant effect.

Although the most destructive manifestation of Mn toxicity is a neurological syndrome of extrapyramidal motor dysfunction resembling Parkinson's disease which is frequently found to develop in workers occupationally exposed to Mn-containing dust (Cook et al., 1974; Mena et al., 1967; Penalver, 1955; Rodier, 1955), other significant biological effects of chronic Mn exposure have been reported. Gubler et al. (1954) produced a microcytic, hypochromic anemia in rats by exposing them to excessive dietary Mn. Interference with normal Cu metabolism was also detected in these rats. Rehnberg et al. (1980) observed an Mn dose-related acceleration of liver Fe depletion as well as various hematological effects in preweanling rats chronically intubated with an Mn_3O_4 suspension. Singh et al. (1974) reported that, histopathologically, the testes were the most vulnerable organs in early Mn

poisoning in the rat. It was because of these reported effects of Mn on non-CNS tissues that the present extensive distribution study was undertaken.

At the dose level used in the present study (3.0 mg Mn/kg), an insignificant increase in hepatic Mn concentration over controls was noted (Table 4.5). These findings are consistent with the fact that, although Mn concentrates in the liver soon after ip or iv administration (Maynard and Cotzias, 1955); it is efficiently and swiftly eliminated from this organ by secretion into the bile. Yet other researchers have reported increased liver Mn levels after chronic exposure. For example, Rehnberg et al. (1980) administered Mn_2O_4 to neonatal rats and found a progressive increase in liver Mn over an experimental period of 20 days. However, in neonates, the physiological mechanisms involved in biliary excretion are not yet fully mature and functional (Klaassen, 1972; Klaassen, 1973). Indeed, Cotzias et al. (1974) observed a complete absence of elimination of injected Mn in rats up to 18 days of age. Thus, progressive hepatic accumulation of Mn in neonates is to be expected, but in adult rats, this should not be the case unless the daily dose of the metal exceeds the capacity of the liver to excrete it effectively. This may explain the results of Singh et al. (1974) who, using a daily ip dose of 6 mg Mn/kg, noted significant increases in liver Mn content as well as focal necrosis in liver tissue. Such damage to the liver may contribute to less effective biliary excretion and consequently increased hepatic retention of the metal.

Maynard and Cotzias (1955) found that, 1/2 hr after a single trace dose of radioactive Mn (^{56}Mn), the highest concentration of ^{56}Mn in the liver was localized in the mitochondrial fraction. This finding has led

to the hypothesis that Mn may produce its toxic effects by interfering with energy metabolism (Singh et al., 1974). However, it is clear from the data of the present study that excess tissue Mn does not concentrate selectively in mitochondria (Fig. 4.9). Thus, the long-term subcellular kinetics of Mn appear to be substantially more complex than what has been observed in single injection studies.

The increase in blood Mn content after Mn treatment was interesting in that most of the increase could be accounted for by the binding of Mn to hemoglobin within erythrocytes (Table 4.2). In contrast, plasma Mn levels were very low in both the control and treated group. The nature of Mn binding to hemoglobin has yet to be determined but a possibility is that at least some of the metal may be bound within the heme rings of the molecule. This possibility is supported by earlier studies which demonstrated the existence of naturally occurring Mn-porphyrins in erythrocytes (Borg and Cotzias, 1958; Hancock and Fritze, 1973) and by the fact that metals other than Fe can be inserted into the heme moiety of hemoglobin under certain conditions. Lamola and Yamane (1974) demonstrated that the fluorescent porphyrin in the erythrocytes of patients with Pb-intoxication or severe Fe-deficiency anemia is Zn-protoporphyrin that is bound to globin moieties, probably at heme binding sites, and Mena et al. (1969) reported that anemia in human subjects was associated with a high intestinal absorption of Mn and a higher level of Mn in erythrocytes as compared to normal controls. Reconstituted hemoglobin in which all Fe has been replaced in vitro by Mn (metmanganoglobin) has been shown to be identical in quaternary structure and similar in tertiary structure to normal hemoglobin (Moffat

et al., 1976). Future experiments designed to further clarify the effects of chronic Mn exposure on hemoglobin structure and synthesis should be undertaken.

The Cu content of several tissues was found to be increased after chronic exposure to Mn (Table 4.5). A concurrent increase in Cu within several brain regions was also observed. As mentioned previously, these tissue increases may be a reflection of plasma increases (Table 4.3). The reason for these plasma increases is obscure. Increased plasma or serum Cu concentrations have been found to be associated with a spectrum of disease conditions including acute and chronic infections, various anemias, "collagen" disorders, hemochromatosis and myocardial infarctions (Underwood, 1977).

The ability of Mn to affect the normal concentrations of Zn and Mg in bone may indicate a direct action of Mn on the incorporation of Zn and Mg into the bones of rats. This speculation is supported by the observation that Mn levels in bone were much increased due to exposure. I am not aware of any reports in the literature describing the effects of chronic Mn excess on bone development although Mn deficiency has long been known to cause skeletal abnormalities in developing animals (Amdur et al., 1945; Everson et al., 1959; Wilgus et al., 1939). These effects have since been shown to be due to abnormalities in the chondrogenic process (Leach, 1968) caused by defects in mucopolysaccharide biosynthesis (Leach, 1971), a process which is dependent upon the presence of Mn.

In the course of this study, it was noted that pancreatic tissue accumulated 3-60x more Mn than other soft tissues (Table 4.5). Concurrently, the Fe content of the pancreas was increased. This latter finding was surprising since 1) there were no changes in the Fe

concentration of other tissues, and 2) it was expected that any change in tissue Fe levels due to chronic Mn administration would be in a negative direction since Mn competes with Fe for binding sites on intestinal uptake proteins and in the presence of high levels of Mn, absorption of Fe may be depressed (Diez-Ewald et al., 1968; Thomson et al., 1971). It was probably due to this Mn-Fe antagonism that Gubler et al. (1954) were able to produce a microcytic, hypochromic anemia accompanied by decreased total body Fe content in rats exposed to high levels of Mn for 120 days. Thus the unexpected increases in pancreatic Fe levels noted in the present study might have been due to increased vascularization and/or hemorrhage in this organ as a consequence of Mn treatment. It was for these reasons that a histological examination of pancreatic tissue from Mn treated rats was performed.

To the author's knowledge, histological studies of pancreatic tissue from Mn-exposed animals have not been published previously. Indeed, most reports of a histologic nature have dealt with the effects of Mn on the brain (Chandra and Srivastava, 1970; Chandra and Shukla, 1978; Pentschew et al., 1963; Shukla and Chandra, 1976). This emphasis is reasonable since the major toxic effect of Mn exposure in humans is the production of a neurological syndrome of gait disorders indicative of injury to the brain's extrapyramidal motor system (Cook et al., 1974; Emara et al., 1971; Mena et al., 1967; Rodier, 1955; Smyth et al., 1973). However, chronic Mn administration to experimental animals does result in significant changes in other organs and these changes often precede those which occur in brain. For example, Singh et al. (1974) noted tiny areas of focal necrosis in the liver and degeneration of approximately 10% of the seminiferous tubules in testes of rats exposed up to 6 mg Mn/kg for 25 days. (This dose was twice that used in the

present study.) In that study, no histopathological changes were noted in the brain, and other organs, including pancreas, were not examined. Some of these toxic effects were found to be enhanced by iron deficiency (Chandra and Tandon, 1973).

In rabbits, exposed intratracheally to a single dose of manganese dioxide (250 mg/kg) and sacrificed at intervals of 2, 4, 6 and 8 months, a progressive destruction of seminiferous tubules accompanied by a corresponding decrease in testicular succinic dehydrogenase activity was observed (Seth et al., 1973). After 2 months, about 10-20% of tubules showed degenerative changes, whereas by 8 months, most of the tubules were collapsed and there was fibrosis and patchy calcification present. Again, other organs were not analyzed but Mn accumulation is greater in the pancreas than in the testes after ip injections (Table 4.5).

Pentschew et al. (1963) administered MnO_2 intramuscularly (5500 mg total dose) to a rhesus monkey which was sacrificed 14.5 months later after developing movement disorders of a neurological nature. In the brain, severe, selective damage to the subthalamic nucleus and medial pallidum was observed. In the liver, hemosiderosis of the Kupffer cells was seen. No histological changes in the kidney, adrenals, heart or lungs were noted. The pancreas was not examined.

Several confounding variables become apparent when an attempt is made to compare the above mentioned studies one with the other or with the present report. There are differences with regard to the experimental animals used, the chemical form of Mn, the dose of Mn, the mode of administration and the total duration of the experiment. In this regard, the present study most closely resembles that of Singh et al. (1974) in which rather minor histological changes were noted in the liver and testes of rats after 25 days of daily ip injections of soluble

Mn (6.0 mg/kg). On the other hand, I observed significant changes in pancreatic tissue at a dose level of only 3.0 mg/kg over a comparable time period suggesting that the pancreas may be more sensitive than liver and testes to the toxic effects of injected Mn.

It is probable that the mode of administration employed in the present study (ip injection) was an important factor influencing the production of the histopathological changes which we observed. If the pancreas is indeed more sensitive to Mn than other visceral organs, then the presence of high levels of Mn in the peritoneal cavity, even for short periods of time each day, might potentiate toxic effects that would not occur so readily with other routes of exposure (e.g. oral). This view is supported by the observation that the pathological reaction seen in the present study appeared for the most part to be progressing "from the outside in" (see Figs. 4.11 and 4.12). The outer portions of the gland were the most severely affected while the central portions generally had a normal appearance. When Mn was administered orally via drinking water, a similar inflammatory reaction was not observed, nor was the accumulation of Mn particularly high when compared to that in the present study (see Chapter 6). It is therefore suggested that ip injection is not the most suitable route of administration for studies of greater than 30 days duration. This is especially true for experiments whose purpose is to elucidate the neurotoxicity of chronically administered Mn. If intraperitoneally injected Mn has a selectively toxic effect on the pancreas as the present results indicate, then any subsequent changes that are noted in brain, particularly those of a subtle biochemical nature, may be difficult to interpret since they may not be due primarily to the neuronal disposition of Mn but rather they may be merely effects secondary to

cellular damage in the pancreas or other organs. Unfortunately, ip injection has been and continues to be a frequently used route of administration in chronic Mn toxicity studies, some of which have been of 120 days or greater duration (Chandra and Srivastava, 1970; Sitaramayya et al., 1974; Shukla et al., 1976; Autissier et al., 1982).

Several studies have examined the response of central neurotransmitter levels to subchronic ip injections of Mn salts. These have almost invariably been "whole brain" rodent studies which have not taken into account regional variations in neurotransmitter concentrations. In such studies, whole brain DA and/or NE have frequently been found to be elevated (Cotzias et al., 1974; Chandra et al., 1979; Shukla and Chandra, 1979; Chandra et al., 1980). The present study confirms and extends these previous findings. By employing a regional approach, it was possible to determine that increases in DA and NE do not occur simultaneously throughout the brain in response to Mn treatment. NE was significantly increased in the pons-medulla but unchanged in the striatum and frontal cortex, whereas DA was increased in the pons-medulla and frontal cortex, but not in the striatum. Levels of the DA metabolites HVA and DOPAC were not significantly different from controls in any of the regions analyzed. The fact that metabolite levels were unchanged indicates that the observed increases in catecholamine concentrations probably do not occur as a result of decreased catabolism but are more likely due to increased rates of synthesis. In support of this contention, cerebral TH activity has been reported to be increased during the early stages of Mn intoxication in rats (Bonilla, 1980). Mn^{2+} , however, has no direct activating influence on TH when added in vitro (Deskin et al., 1980), therefore increased TH activity and increased catecholamine levels must occur as secondary effects of the

actions of Mn at some other level. In this regard, it should be remembered that, because of the complexity of neuronal interconnections within the brain, the activity of neurons which use a particular neurotransmitter may be influenced either directly or indirectly by neurochemical changes elsewhere in the brain (e.g., see Fig. 1.2). Thus the increased levels of DA and NE observed in this study and others may well be secondary effects due to the action of Mn on some other neurotransmitter system such as that of a GABA-ergic or cholinergic nature.

It is interesting that changes in DA were noted in brain regions with little or no dopaminergic innervation, whereas in the corpus striatum, a region rich in dopaminergic nerve terminals, no changes were observed. Also, DA-receptor density in the corpus striatum was unchanged due to Mn administration. It is therefore suggested that in the early stages of Mn intoxication, the major extrapyramidal dopaminergic tracts may not be affected by elevated Mn levels. In this regard, it may be significant to note that the first manifestations of Mn poisoning in humans are often psychological (Mn psychosis) in nature with the absence of neurological movement disorders (Mena et al., 1967; Emara et al., 1971). Thus, it is suggested that perhaps future studies of subchronic Mn administration to experimental animals should concentrate on characterizing changes in dopaminergic parameters in the limbic system (nucleus accumbens, septum, amygdala) rather than in the extrapyramidal motor system.

Table 4.1

Dry Weights of Several Rat Brain Regions

Values are means \pm S.E., $n = 23$.

Brain Region	Dry Weight (mg)
1. Olfactory bulb	14.0 \pm 0.8
2. Frontal cortex	37.1 \pm 1.6
3. Rest of cortex	49.7 \pm 2.3
4. Corpus callosum	13.1 \pm 0.7
5. Hippocampi	23.0 \pm 0.7
6. Amygdalae	13.3 \pm 0.5
7. Corpus striatum	17.4 \pm 0.5
8. Colliculi (tectum)	10.8 \pm 0.4
9. Midbrain tegmentum	14.0 \pm 0.6
10. Thalami	23.0 \pm 1.0
11. Hypothalamus	6.3 \pm 0.2
12. Pons-medulla	53.4 \pm 1.4
13. Cerebellum	55.8 \pm 1.3

Table 4.2

Mn in Blood

Effect of subchronic Mn exposure on the levels of Mn in blood. Values are means \pm S.D.

^a $\mu\text{g Mn/ml}$

^b ng Mn/ml

^c $\mu\text{g Mn/g}$

	Control. (n=4)	Mn Treated (n=5)
^a Whole Blood	< 0.04	0.16 \pm 0.04
^b Plasma	1.7 \pm 0.2	5.0 \pm 1.5
^a RBC	< 0.04	0.28 \pm 0.08
^c Dry RBC	0.10 \pm 0.07	0.48 \pm 0.12
^c Dry Dialyzed Hemolysate	0.40 \pm 0.16	1.01 \pm 0.12

Table 4.3

Blood Metal Content

Effects of subchronic Mn exposure on the blood levels of Zn; Fe and Cu.

Values are means \pm S.D., n=5. C = control; T = Mn treated.

(*)Significantly different from control, $p < 0.05$.

		Plasma ($\mu\text{g/ml}$)	Dry RBC ($\mu\text{g/g}$)
Zn	C	1.4 ± 0.3	27.5 ± 2.6
	T	$1.0 \pm 0.3(*)$	26.6 ± 1.6
Fe	C	2.7 ± 0.9	2927 ± 405
	T	2.4 ± 0.8	2982 ± 182
Cu	C	1.4 ± 0.2	3.5 ± 0.7
	T	$2.2 \pm 0.5(*)$	3.2 ± 0.5

Table 4.4

Effects of Subchronic Mn Administration on the
Distribution of Essential Trace Metals in Rat Brain

Values are means \pm S.D., n = 6

(*) Significantly different from control, p < 0.05

C = Control; T = Mn-treated

	Cu (nmol/g dry wt.)	Zn (μ mol/g dry wt.)	Fe (μ mol/g dry wt.)
Olfactory bulb	C 152 \pm 23 T 161 \pm 45	1.10 \pm 0.04 1.12 \pm 0.09	1.38 \pm 0.06 1.46 \pm 0.16
Frontal cortex	C 127 \pm 15 T 165 \pm 22(*)	1.23 \pm 0.07 1.24 \pm 0.04	1.08 \pm 0.09 1.06 \pm 0.10
Rest of cortex	C 132 \pm 9 T 179 \pm 18(*)	1.23 \pm 0.05 1.21 \pm 0.06	1.04 \pm 0.05 1.04 \pm 0.06
Corpus callosum	C 99 \pm 19 T 175 \pm 48(*)	0.62 \pm 0.03 0.58 \pm 0.03	0.84 \pm 0.07 0.87 \pm 0.09
Hippocampus	C 154 \pm 9 T 183 \pm 14(*)	1.15 \pm 0.08 1.09 \pm 0.08	1.11 \pm 0.20 1.03 \pm 0.09
Amygdala	C 126 \pm 10 T 240 \pm 114(*)	1.58 \pm 0.08 1.44 \pm 0.06(*)	1.17 \pm 0.07 1.18 \pm 0.11
Corpus striatum	C 159 \pm 14 T 208 \pm 26(*)	0.90 \pm 0.04 0.82 \pm 0.06	1.28 \pm 0.06 1.20 \pm 0.07
Colliculi	C 152 \pm 14 T 288 \pm 144	0.83 \pm 0.14 0.72 \pm 0.04	1.47 \pm 0.14 1.54 \pm 0.27
Midbrain	C 148 \pm 17 T 183 \pm 34	0.60 \pm 0.02 0.56 \pm 0.04	1.10 \pm 0.11 1.06 \pm 0.11
Thalamus	C 140 \pm 12 T 191 \pm 31(*)	0.65 \pm 0.02 0.61 \pm 0.09	0.99 \pm 0.06 0.93 \pm 0.09
Hypothalamus	C 240 \pm 30 T 305 \pm 84	0.90 \pm 0.05 0.80 \pm 0.08(*)	1.75 \pm 0.20 1.75 \pm 0.34
Pons-medulla	C 84 \pm 10 T 115 \pm 22(*)	0.59 \pm 0.04 0.57 \pm 0.04	0.83 \pm 0.10 0.74 \pm 0.04
Cerebellum	C 183 \pm 14 T 220 \pm 24(*)	0.84 \pm 0.05 0.83 \pm 0.05	1.09 \pm 0.04 1.09 \pm 0.04

Table 4.3

Tissue Mn, Mg, Zn, Fe and Cu Content^a

Effect of subchronic Mn exposure on tissue concentration of metals.
Tissues are listed in order of decreasing Mn content in controls.
Values are means \pm S.D. (n=6).

^a $\mu\text{g/g dry wt.}$

C = control; T = Mn treated

*Significantly different from control, $p < 0.05$.

		Mn		Mg		Zn		Fe		Cu
Pancreas	C	6.72 \pm 1.37		922 \pm 122		76 \pm 11		86 \pm 38		3.0 \pm 0.6
	T	38.78 \pm 17.68*		1154 \pm 100		83 \pm 13		163 \pm 29*		5.0 \pm 0.8*
Liver	C	5.25 \pm 1.17		819 \pm 47		111 \pm 4		402 \pm 54		21.1 \pm 1.3
	T	7.21 \pm 2.27		797 \pm 96		122 \pm 20		386 \pm 62		22.0 \pm 2.0
Duodenum	C	1.83 \pm 0.32		964 \pm 16		105 \pm 6		108 \pm 18		5.7 \pm 0.5
	T	11.01 \pm 3.51*		935 \pm 104		101 \pm 16		108 \pm 29		6.4 \pm 0.6*
Kidney	C	1.68 \pm 0.26		918 \pm 92		98 \pm 9		253 \pm 40		32.2 \pm 5.3
	T	6.58 \pm 1.95*		978 \pm 63		96 \pm 6		280 \pm 48		34.3 \pm 6.3
Testes	C	0.75 \pm 0.07		1235 \pm 95		187 \pm 14		130 \pm 26		9.1 \pm 0.4
	T	4.86 \pm 1.87*		1216 \pm 108		187 \pm 11		122 \pm 17		10.5 \pm 1.2*
Heart	C	0.47 \pm 0.05		1137 \pm 91		81 \pm 8		440 \pm 96		19.4 \pm 1.5
	T	1.50 \pm 0.40*		1030 \pm 32*		77 \pm 4		459 \pm 14		21.6 \pm 3.1
Bone (femur)	C	0.43 \pm 0.04		5910 \pm 273		214 \pm 9		66 \pm 9		1.3 \pm 0.1
	T	37.84 \pm 9.59*		5471 \pm 177*		186 \pm 14*		61 \pm 19		1.8 \pm 0.2*
Spleen	C	0.34 \pm 0.07		1044 \pm 68		99 \pm 9		1018 \pm 124		4.4 \pm 1.1
	T	1.33 \pm 0.71*		1047 \pm 61		101 \pm 9		970 \pm 138		5.3 \pm 0.3
Lung	C	0.28 \pm 0.03		786 \pm 67		107 \pm 1		414 \pm 29		6.2 \pm 0.3
	T	1.04 \pm 0.26*		714 \pm 44		103 \pm 5		410 \pm 20		7.3 \pm 0.7*
Skeletal Muscle	C	0.15 \pm 0.02		1240 \pm 88		38 \pm 3		44 \pm 3		4.5 \pm 0.3
	T	0.57 \pm 0.07*		1327 \pm 73		43 \pm 6		43 \pm 4		4.8 \pm 0.5

Table 4.6

Increases in Tissue Mn Levels

Increases in tissue Mn concentrations due to subchronic Mn exposure. Values are expressed as multiples of the corresponding control Mn concentration.

TISSUE	Mn INCREASE
Liver	1.0 - 1.5 x
Heart	
Lung	
Kidney	3 - 4 x
Spleen	
Brain	
Skeletal Muscle	
Pancreas	
Testes	~ 6 x
Duodenum	
Bone	85 - 90 x

Table 4.7

The Effects of Subchronic Mn Administration on
Neurochemical Parameters in the Frontal Cortex

(*) Significantly different from control, $p < 0.05$

^a ng/g wet wt.

^b Values are means \pm S.D., $n = 6$

	Control	Mn Treated
^a DA	^a 43.8 \pm 10.2	139.6 \pm 94.5(*)
DOPAC	39.7 \pm 8.3	42.7 \pm 10.9
HVA	60	60
NE	212 \pm 25	218 \pm 29
5-HT	216 \pm 30	210 \pm 18
5-HIAA	149 \pm 34	130 \pm 23
Mn	250 \pm 110	670 \pm 90(*)

Table 4.8

The Effects of Subchronic Mn Administration on
Neurochemical Parameters in the Pons-Medulla

(*) Significantly different from control, $p < 0.05$

^a ng/g wet wt.

^b Values are means \pm S.D., $n = 6$

	Control		Mn Treated	
^a DA	^b 28.7	\pm 1.9	56.4	\pm 25.9(*)
/ DOPAC	24.7	\pm 3.8	23.0	\pm 5.8
HVA	16.5	\pm 3.6	17.2	\pm 8.3
NE	166	\pm 13	221	\pm 29(*)
5-HT	1404	\pm 496	1204	\pm 357
5-HIAA	993	\pm 419	790	\pm 262
Mn	640	\pm 150	1700	\pm 170(*)

Table 4.9

The Effects of Subchronic Mn Administration on
Neurochemical Parameters in the Corpus Striatum

(*) Significantly different from control, $p < 0.05$

^a ng/g wet wt.

^b Values are means \pm S.D., $n = 6$

^c Stereospecifically bound [³H]-SPIRO, fmol/mg wet tissue

	Control		Mn Treated	
^a DA	4560	\pm 768	4416	\pm 375
DOPAC	4629	\pm 581	4369	\pm 488
HVA	894	\pm 134	855	\pm 167
NE	283	\pm 67	263-	\pm 50
5-HT	161	\pm 106	202	\pm 112
5-HIAA	615	\pm 262	515	\pm 202
Mn	560	\pm 160	1760	\pm 220(*)
^c DA Receptors	17.6	\pm 3.7	16.4	\pm 3.3

FIGURES

Figure 4.1 Whole rat brain, dorsal and ventral views. Brain regions indicated are: 1, olfactory bulb; 2, frontal cortex; 3, rest of cortex; 6, amygdala-pyriform cortex; 9, tegmentum; 11, hypothalamus; 12, pons-medulla; 13, cerebellum. "A" indicates the level of section for Fig. 4.2

Figure 4.2 Frontal section at level "A" from Fig. 4.1 and 4.3. l.v., lateral ventricle; a.c., anterior commissure; r.f., rhinal fissure, o.c., optic chiasm. Brain regions indicated are: 3, rest of cortex; 4, corpus callosum; 6, amygdala; 7, corpus striatum; 11, hypothalamus. "C" indicates level of section for Fig. 4.3.

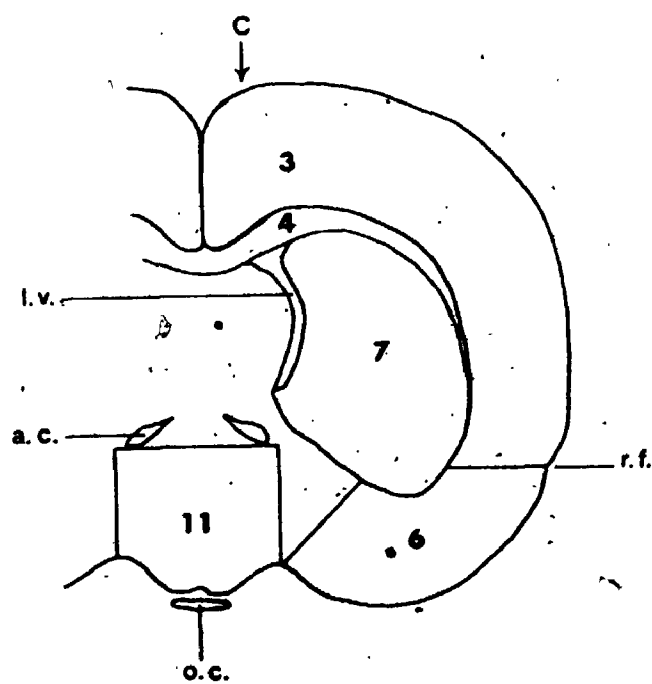
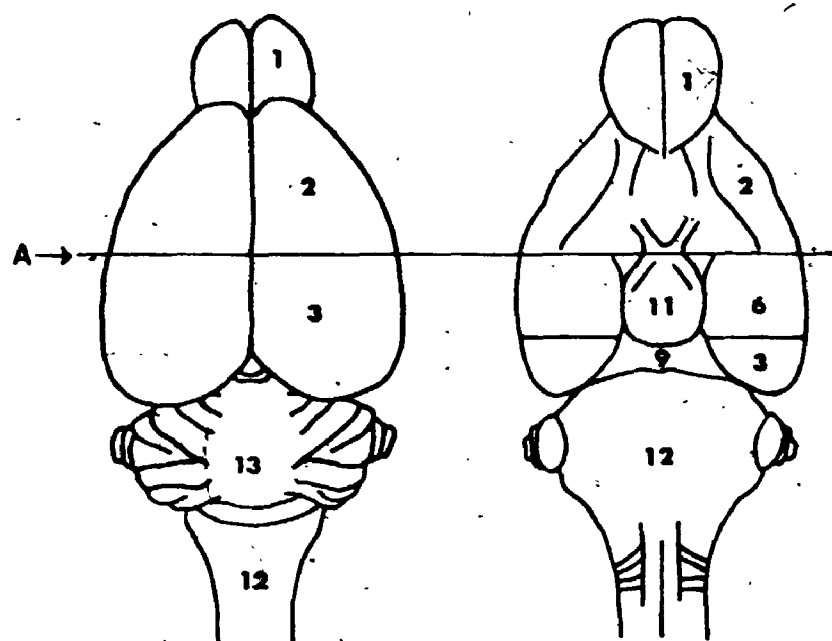


Figure 4.3

Parasagittal section at level "C" from Fig. 4.2

showing relative positions of several brain regions.

Numbered regions are as listed in Table 4.1. "A"

indicates the level of section for Fig. 4.2; "B" for
Fig. 4.4.

Figure 4.4

Horizontal section at level "B" from Fig. 4.3.

Numbered regions are as listed in Table 4.1.

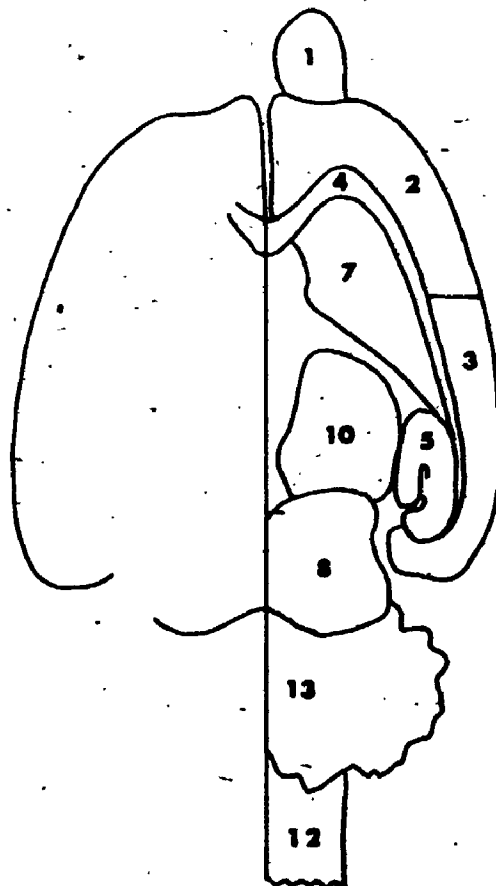
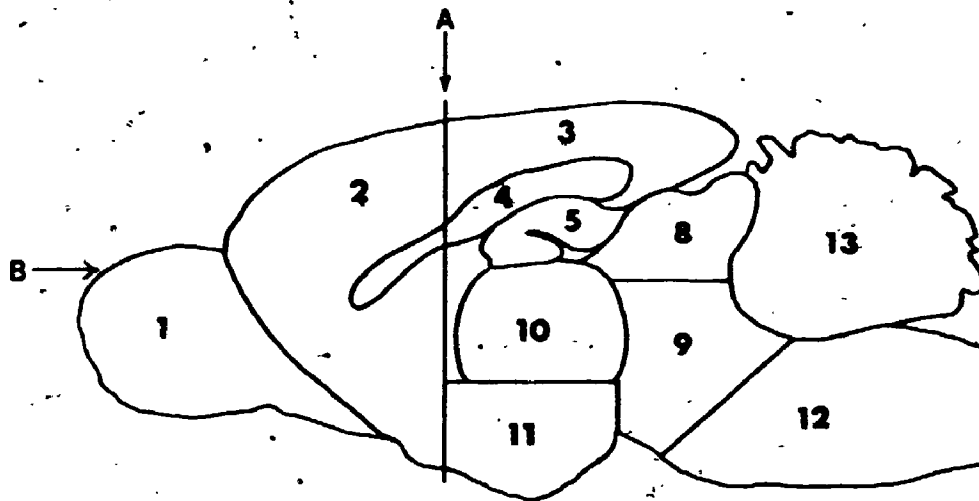


Figure 4.5

Effect of subchronic Mn administration on the Mn concentration of 13 brain regions. 1, Olfactory bulb; 2, frontal cortex; 3, rest of cortex; 4, corpus callosum; 5, hippocampus; 6, amygdala; 7, corpus striatum; 8, colliculi; 9, midbrain tegmentum; 10, thalamus; 11, hypothalamus; 12, pons-medulla; 13, cerebellum. Each value represents the mean \pm S.D. of six rats.

Figure 4.6

Relationship between Mn and Fe distribution in normal rat brain. Numbered points represent average values for control rats ($n = 6$) from Fig. 4.5 (Mn) and Table 4.4 (Fe). Numbers correspond to the order of brain regions as listed in Fig. 4.5. $r = 0.81$, $p < 0.01$.

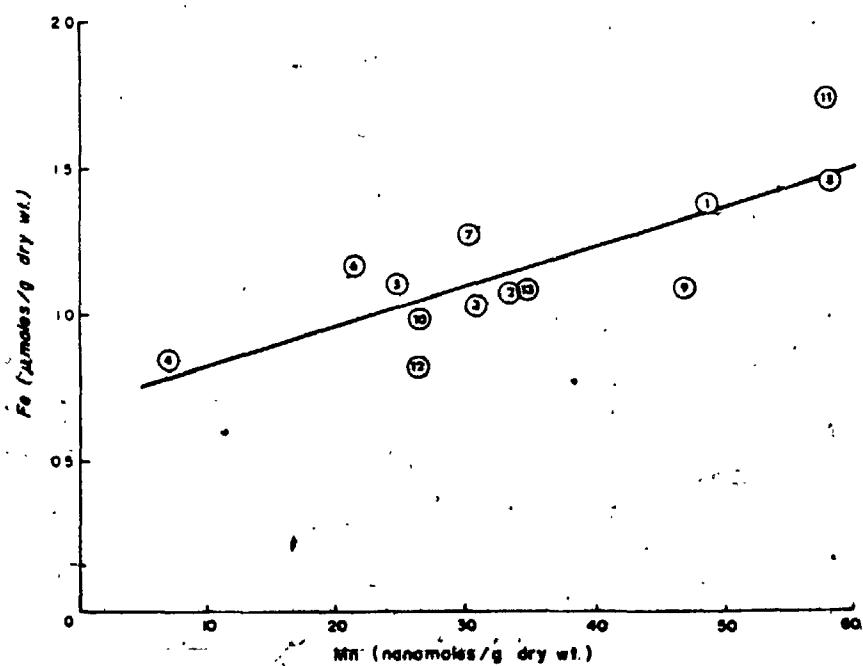
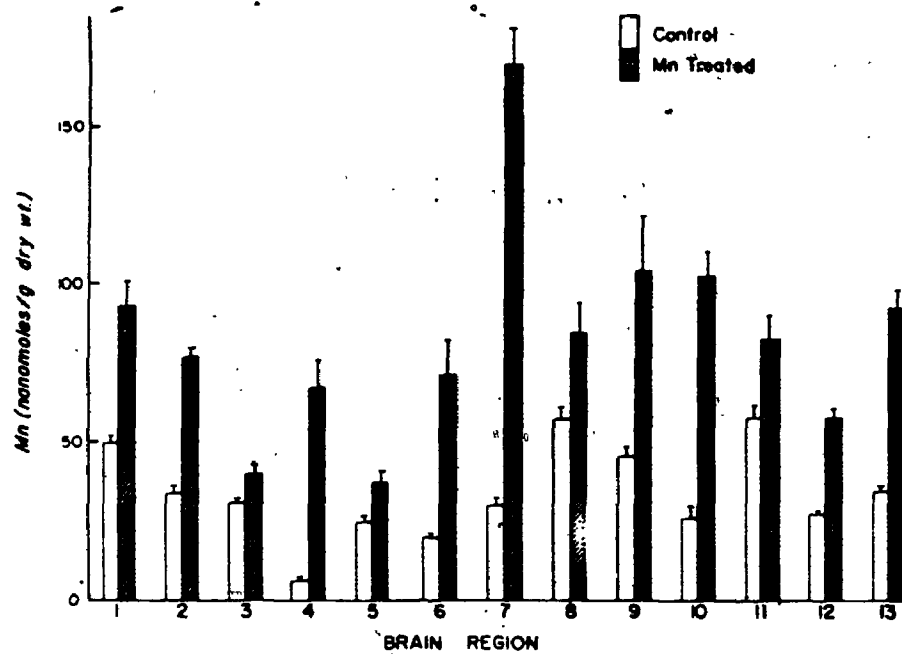


Figure 4.7

Relationship between Mn and Cu distribution in normal rat brain. Numbered points represent average values for control rats ($n = 6$) from Fig. 4.5 (Mn) and Table 4.4 (Cu). Numbers as in Fig. 4.6. $r = 0.66$, $p < 0.05$.

Figure 4.8

Relationship between Mn and Zn distribution in normal rat brain. Numbered points represent average values for control rats ($n = 6$) from Fig. 4.5 (Mn) and Table 4.4 (Zn). Numbers as in Fig. 4.6. $r = -0.05$, not significant.

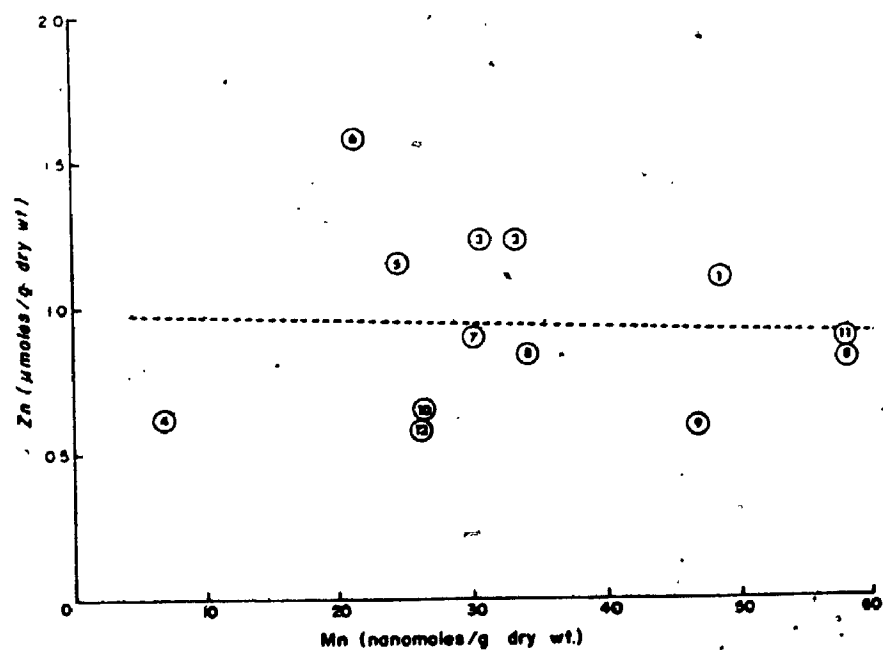
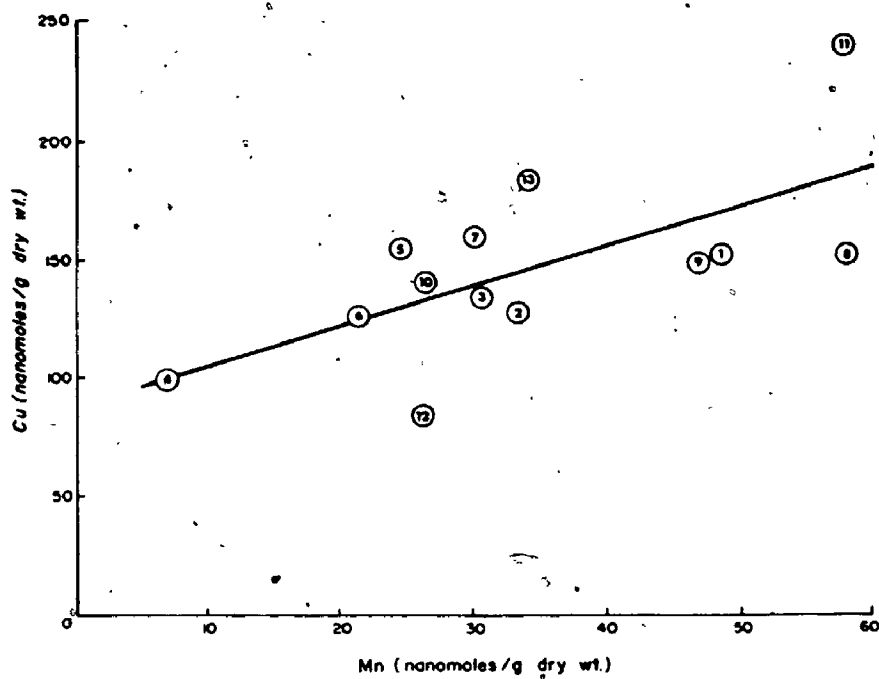


Figure 4.9

Effect of subchronic Mn exposure on the subcellular distribution of Mn in kidney. Open bars = control; hatched bars = Mn treated. Values are means \pm S.D.,

n = 4.

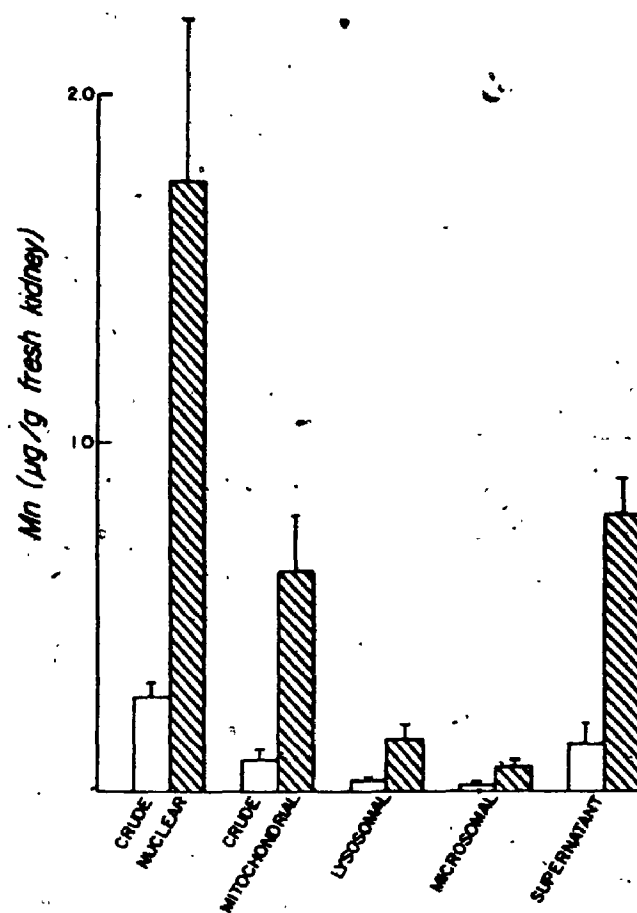
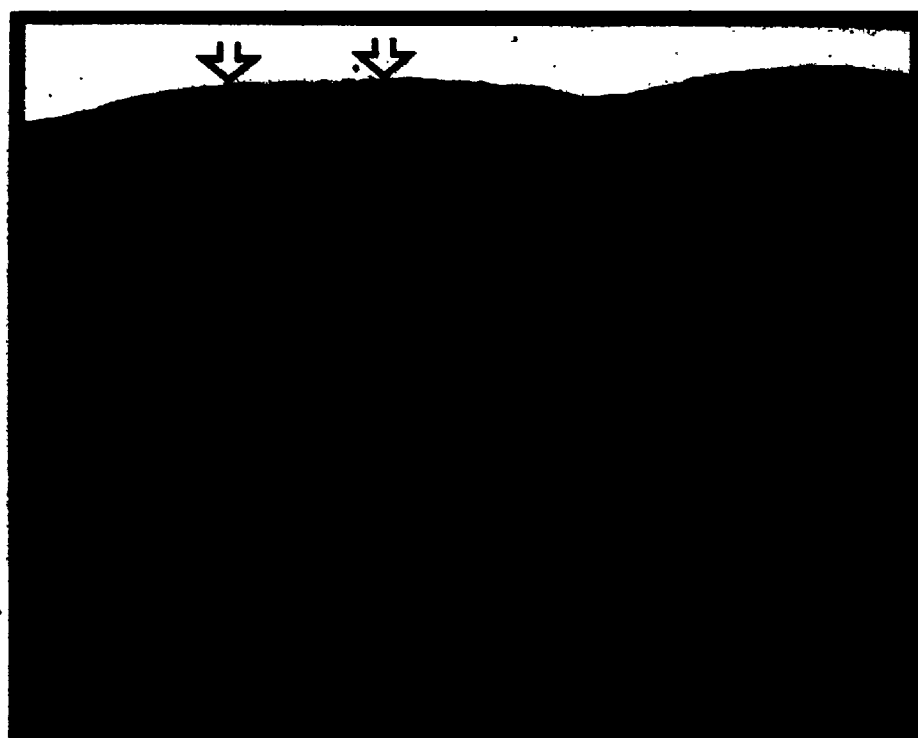


Figure 4.10

Photomicrographs of control rat pancreas. (i) Islet of Langerhans, (d) duct, (small arrows) acini. (a) Note close packing of acini, thin delicate connective tissue capsule (open arrows) and interlobular septa (closed arrows). H & E, X100. (b) Higher magnification of boxed area from (a). H & E, 250X.



a



b

Figure 4.11 . Pancreas from Mn-exposed rat. Note thickened connective tissue capsule and fibrotic invagination (CT), and expanded interacinar spaces (arrows). H & E, 100X.

Figure 4.12 . Pancreas from Mn exposed rat showing acini in various stages of disintegration (arrows) surrounded by fibrotic connective tissue (CT). Note also expanded interacinar spaces in top left of field. H & E, 310X.



Figure 4.13

Pancreas from Mn-exposed rat. (a) Note thick mass of connective tissue (CT) surrounding "islands" of degenerating acini (arrows). H & E, 125X. (b) Higher magnification of boxed area from (4a). Note normal appearance of Islet of Langerhans in close proximity to degenerating acini (arrows). H & E, 400X.

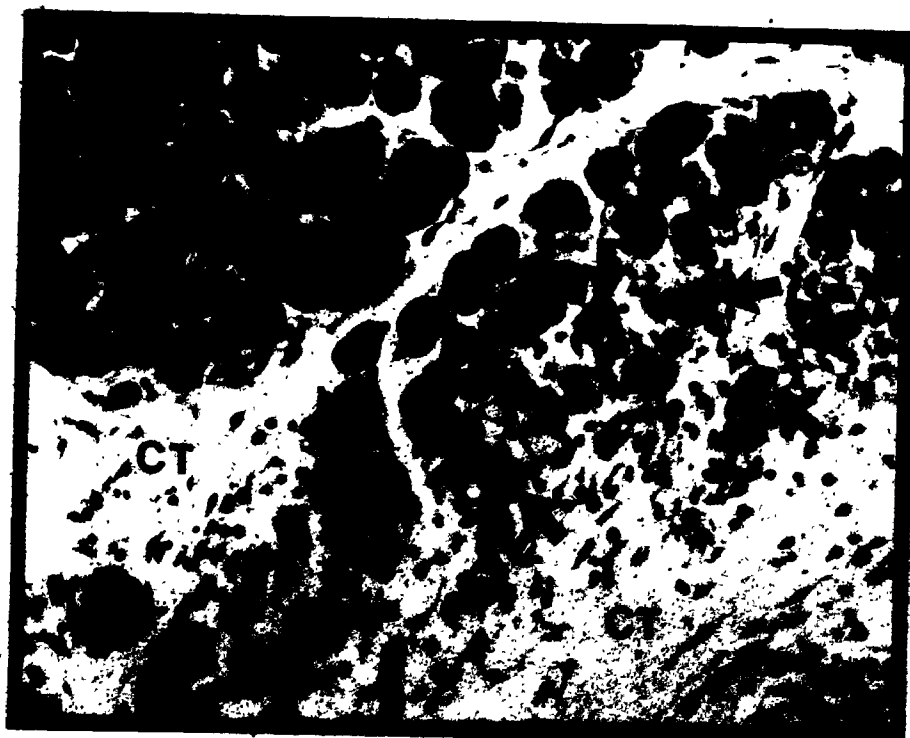


Figure 4.14

Actual chromatograph of 3 ng each of the indicated standards prepared in 0.1 M HClO_4 /1.0 mM EDTA. Bracketted values are elution times (minutes.seconds) after injection. Mobile phase: 0.07 M phosphate/1.0 mM EDTA/0.5 mM $\text{NaCH}_3(\text{CH}_2)_7\text{OSO}_3$, pH 3.8, 8% methanol; flow rate = 2.0 ml/min. Volume injected = 30 μl ; chart recorder speed = 2.5 mm/min.

FIGURE 4.14

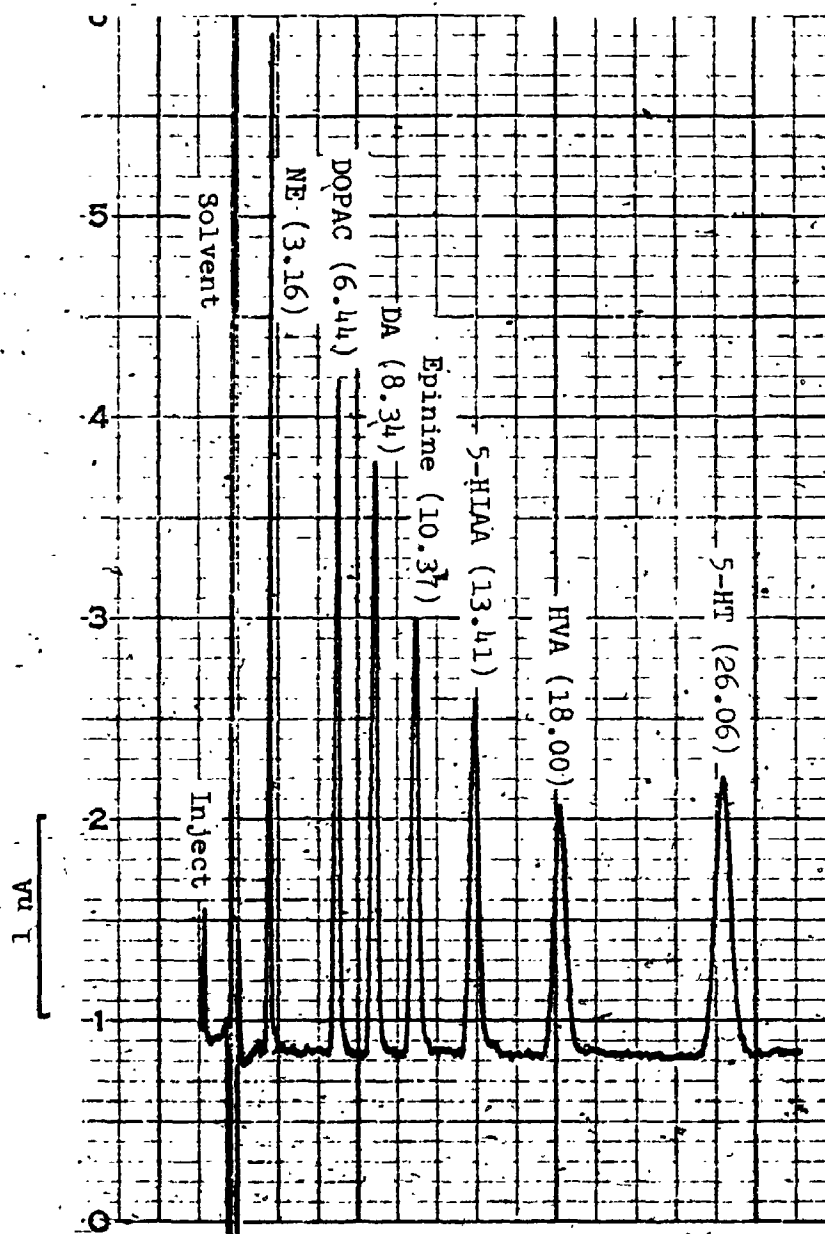
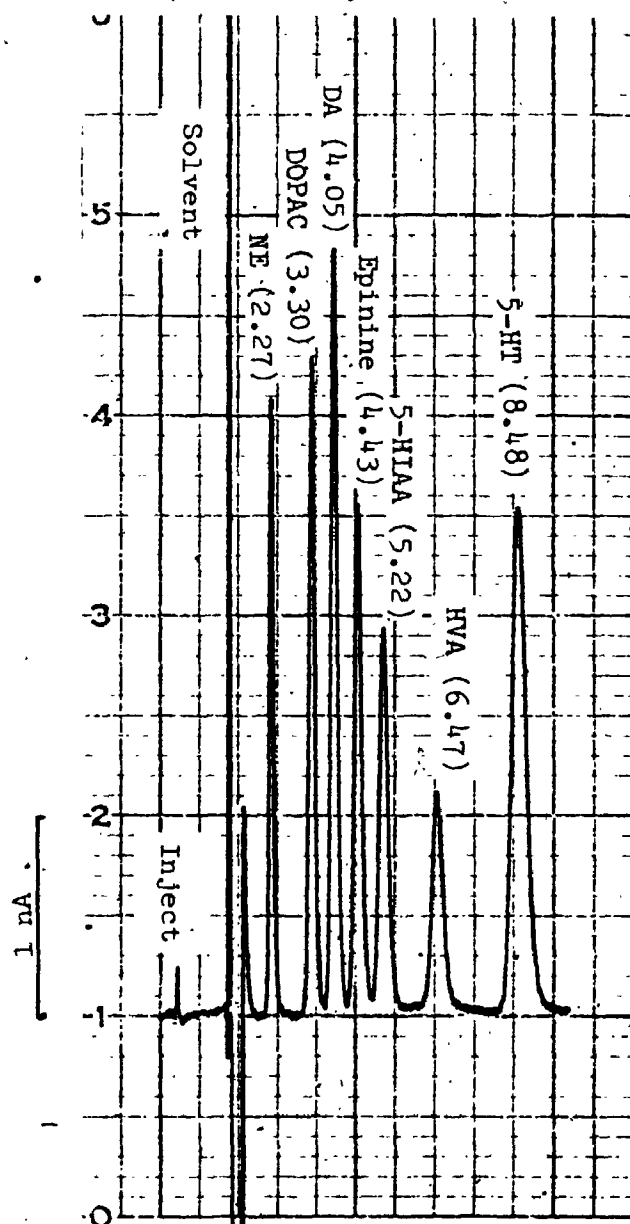


Figure 4.15

Actual chromatograph, conditions as in Fig. 4.14 but 16% methanol in mobile phase instead of 8%, chart recorder speed = 5.0 mm/min. The major effect of the additional methanol is to significantly decrease elution times.

FIGURE 4.15



CHAPTER FIVE

THE ELIMINATION OF EXCESS Mn AFTER SUBCHRONIC INJECTION

5.1 Methods

5.1.1 Animals and Treatment

Four groups of male Sprague-Dawley rats (75-100 g) were given access to standard chow and tap water ad libitum. Groups 2, 3, and 4 received daily ip injections of Mn (3.5 mg/kg Mn as $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ in .9% NaCl) for 30 days. Group 1 served as control and received equal volumes of saline only. At the end of 30 days, treatment was terminated and a 48 hr period was allowed for the excretion of any unbound Mn at which time all rats of Group 1 and 2 were sacrificed by aortal exsanguination under light pentobarbital anesthesia. Brain, liver, kidney, testes and skeletal muscle were removed for metal analysis. Meanwhile, animals of Groups 3 and 4 were placed in separate metabolic cages. Group 3 received no further treatment, but Group 4 received daily ip injections of CaNa_2EDTA (50 mg/kg in .9% NaCl) for five days. Treatment was discontinued for two days and then resumed for an additional five days. Urine was collected daily for analysis of metal content. Fourteen days after initiating post-Mn treatment, all rats from Groups 3 and 4 were sacrificed and tissues removed as described for Groups 1 and 2. Tissues were prepared for metal analysis as detailed in section 3.2.

5.1.2 Statistical Analysis

Where applicable, the four groups were compared by analysis of variance. If significant f-values were obtained, groups were further compared by Student's t-test. Also, correlation analyses were performed to determine whether there were relationships between Mn excretion and the excretion of other heavy metals. The reference text for the statistical methods was Sokal and Rohlf 1969.

5.2 Results

5.2.1 Urinary Excretion of Metals

EDTA treatment increased the urinary excretion of Mn, Zn, and Fe, but not Cu, in Mn-exposed rats (Fig. 5.1); this increase occurred despite a decrease in daily urine volume. Mn excretion increased approximately 12X due to treatment, Zn 6X, Fe 2X, and Cu excretion decreased 0.2X. The time-course profile of Mn excretion is given in Fig. 5.2. The normal urinary excretion of Mn was extremely low (~ 0.5 $\mu\text{g/day}$) even though rats had been chronically exposed to Mn. There was an excellent positive correlation between urinary Mn and urinary Fe excretion for EDTA-treated rats ($r = 0.74$, $p < 0.01$). Linear regression analysis of the data points revealed that $\mu\text{mol Fe excreted per day} = 0.02 + 0.92 \mu\text{mol Mn}$, therefore approximately 1 mole of Fe was excreted for each mole of Mn. No significant correlations were obtained between Mn and Zn ($r = 0.34$, n.s.) or Mn and Cu ($r = 0.22$, n.s.) excretion.

5.2.2 Tissue Metal Concentrations

Tissue Mn concentrations increased significantly in kidney, testes and skeletal muscle, but not in liver, due to administration of MnCl_2 (Fig. 5.3). Upon cessation of treatment, excess Mn was spontaneously eliminated from all tissues. After two weeks, kidney levels had almost returned to within the normal range. Levels were significantly reduced in testes and muscle. Ten days of EDTA treatment did not enhance the elimination of Mn from tissues except in muscle. The hepatic Mn concentration increased after termination of MnCl_2 administration.

Of the three brain regions examined, accumulation of Mn varied from 4.0 $\mu\text{g/g}$ above control levels in the hippocampus to 6.7 $\mu\text{g/g}$ above control in the tegmentum after 30 days of Mn administration (Fig. 5.4). Two weeks after the cessation of Mn treatment, levels had fallen significantly, but they remained substantially higher than control values. EDTA administration had no significant effect on the natural elimination of excess Mn from brain tissue. Changes in tissue Zn, Fe and Cu levels are presented in Table 5.1. Little change in tissue Zn concentrations were observed in response to EDTA treatment. EDTA caused increased levels of Zn in the kidneys, but this was not remarkable considering the high levels of Zn being excreted by this organ during EDTA administration. An increase in the concentration of Cu in testes due to Mn treatment was observed, but levels had returned to normal two weeks after cessation of treatment.

In the brain, no significant differences were noted in Zn or Fe levels among the four groups, but changes in Cu concentrations were observed (Table 5.2). Cu increased in the striatum and hippocampus due to Mn treatment and subsequently remained higher than controls with or without EDTA.

5.3 Discussion

Cotzias et al. (1968) found that neurological signs in Mn-intoxicated miners often persisted after removal from the source of exposure even though tissue Mn levels fell rapidly to within the normal range. It was concluded that chelation therapy would probably be ineffective in correcting the debilitating symptoms of Mn poisoning. Subsequently, L-dopa therapy was used with some success in the treatment of chronic Mn intoxication (Mena et al., 1970). However, Cook et al. (1974) concluded that, in the absence of rigidity or dystonia, L-dopa therapy was not an effective mode of treatment for Mn poisoning. They were able to elicit improvements, sometimes very dramatic, by employing EDTA chelation therapy. Four out of six of their patients who had contracted Mn poisoning while working in an ore crushing plant showed improved postural stability, less facial masking, and decreased bradykinesia after EDTA therapy. Unfortunately, symptoms usually returned after cessation of treatment. The reasons for this relapse were not determined. However, Rodier (1955) noted increased levels of Mn in the hair of 3 patients who had not worked in mines for 2-17 years,

and Flinn et al. (1940) reported a large amount of Mn in the lungs at autopsy of an individual who had suffered from chronic Mn intoxication. Thus, it may be that reservoirs of excess Mn, most likely localized in the lung, may remain even long after the victims of poisoning have been removed from the source of exposure. Therefore, the development of effective, non-toxic chelating agents for use in combating Mn intoxication may yet be a worthwhile endeavour. In this regard, it has been shown in experimental animal studies that polyaminocarboxylic acids were far more effective than thiol chelating agents in their ability to mobilize Mn from vital organs (Tandon and Khandelwal, 1982a) and also in their ability to protect against the lethal effects of acute $MnCl_2$ injections (Tandon and Khandelwal, 1982b). CDTA (1,2-cyclohexylene diamine) and HEDTA (N-(2hydroxyethyl)-ethylenediamine triacetic acid) were particularly effective.

It has been shown in this study that although EDTA injections substantially increased the urinary excretion of Mn in Mn-exposed rats, it did not affect the normal elimination rate of excess tissue Mn. Urinary Zn excretion was also increased, yet, again, tissue concentrations of Zn generally remained unaltered. Millar et al. (1954) reported similar results with regard to EDTA treatment and tissue Zn content. The inability of EDTA to significantly affect tissue concentrations of essential metals while at the same time greatly enhancing urinary excretion of these metals requires further explanation. It may be that a compensatory increase in the intestinal absorption and/or a decrease in the biliary excretion of metals occurs during EDTA treatment, or alternatively, metals may be chelated from other tissues which were not sampled in the present investigation. In

this regard, bone represents a substantial pool of Zn and of Mn in Mn-injected rats (Chapter 4). Mn, which is almost exclusively eliminated from the body via the biliary route under normal conditions (Papavasiliou et al., 1966), increased significantly in the livers of EDTA-treated rats (Fig. 5.3). This may indicate a greater retention and a decreased excretion of Mn by the liver. Also, EDTA is a water-soluble compound and, as such, cannot readily enter the intracellular compartment. As a result, metals must be chelated from plasma, extracellular tissue fluid, and acellular connective tissue, but not from within cells. If essential metals (Mn, Zn, Fe) lost by EDTA chelation can be adequately replaced by mechanisms of increased dietary absorption and/or decreased biliary excretion, there may not be any long lasting loss of intracellular metal content. The net change within the highly cellular tissues such as kidney and liver would be minimal. Our findings are consistent with this explanation. It may be significant that in skeletal muscle, a less cellular tissue than liver and kidney, the decrease in Mn (and Fe) following withdrawal from chronic Mn exposure was significantly greater in the EDTA-treated group (Fig. 5.3; Table 5.1).

A highly significant positive correlation was found in the distribution patterns of endogenous Mn and Fe within brains of normal rats (Chapter 4). Herein, it was noted that a similar correlation exists in the pattern of urinary excretion of these two elements under the influence of EDTA administration, which suggests that the availability of chelatable Mn in the body is related in some manner to the availability of chelatable Fe. This speculation is further

supported by the observation that the EDTA-induced decrease in the Mn content of skeletal muscle was accompanied by a concurrent decrease in Fe (Table 5.1).

In brain, it was shown (Chapter 4) that, of 13 discrete brain regions, the accumulation of chronically administered Mn was higher than average in the tegmentum, striatum, thalamus, amygdala, and corpus callosum. The results of the present study confirm that the tegmentum and striatum accumulated more Mn than the hippocampus (Fig. 5.4). As in other tissues, elimination of excess Mn was spontaneously initiated in all three brain regions after discontinuance of Mn treatment. Thus, the suggestion by Dastur et al. (1971) that CNS tissue is unable to discharge Mn must be modified. Their suggestion was based on the observation that a trace amount of ⁵⁴Mn had an extremely long half-life in the brain. However, the results of the present study indicate that all tissues, including brain, have the ability to increase the turnover of Mn under conditions of increased exposure.

The fluctuations in brain Cu concentrations (Table 5.2) may be important if Mn-induced neurotoxicity is to be understood. Cu levels in the striatum and hippocampus increased significantly due to Mn treatment and remained higher than control values two weeks after discontinuance of Mn injections. Since excessive brain Cu levels, such as occur in Wilson's disease, are known to be neurotoxic, further studies should be aimed at clarifying the mechanism by which Mn exposure induces an elevation in brain Cu in order to determine whether or not this is an important factor in the pathogenesis of Mn neurointoxication.

Table 5.1

The Effect of EDTA Treatment on Tissue Metal
Concentrations After Subchronic Mn Administration

Changes in Zn, Fe and Cu ($\mu\text{g/g}$ wet wt.) after 3.5 mg Mn/kg/day for 30 days, then either no further treatment, or EDTA (50 mg/kg/day ip for 10 days).

Values are means \pm S.D., $n = 5$
(*) Significantly different, $p < 0.05$

		Post-Mn Treatment			
		None		EDTA	
Liver	Zn	27.8	\pm 3.5	29.7	\pm 4.1
	Fe	139.6	\pm 12.4	123.5	\pm 10.8
	Cu	4.4	\pm 0.2	4.6	\pm 0.5
Kidney	Zn	15.3	\pm 1.6	23.7	\pm 4.0(*)
	Fe	64.3	\pm 8.4	60.6	\pm 13.1
	Cu	5.5	\pm 0.9	4.9	\pm 0.7
Testes	Zn	18.7	\pm 3.8	20.4	\pm 2.7
	Fe	10.1	\pm 0.5	10.2	\pm 0.8
	Cu	1.6	\pm 0.4	1.4	\pm 0.2
Skeletal muscle	Zn	7.1	\pm 0.7	6.4	\pm 1.2
	Fe	7.9	\pm 0.8	5.9	\pm 1.7(*)
	Cu	0.72	\pm 0.09	0.63	\pm 0.08

Table 5.2

Changes in brain Cu concentrations ($\mu\text{g/g}$ dry wt.). Group 1, control; Group 2, 3.5 mg/kg/day for 30 days; Group 3, Mn as in Group 2 with no additional treatment; Group 4, Mn as in Group 2, then 50 mg EDTA/kg/day for 10 days subsequent to discontinuance of Mn treatment.

(*) Significantly different from Group 1; all other group comparisons are not significant ($p < 0.05$); $n = 5$ except Group 2, $n = 6$

	Group 1 (control)	Group 2 (Mn treated)	Post-Mn treatment	
			Group 3 (none)	Group 4 (EDTA)-
Midbrain (tegmentum)	8.9 ± 1.5	10.4 ± 1.5	10.6 ± 1.4	10.9 ± 2.6
Corpus striatum	9.8 ± 0.7	$13.9 \pm 2.7(*)$	$13.4 \pm 3.1(*)$	$14.6 \pm 1.7(*)$
Hippocampus	8.0 ± 0.7	$10.2 \pm 0.7(*)$	$10.1 \pm 0.8(*)$	$12.8 \pm 3.3(*)$

FIGURES

Figure 5.1

Average daily volume of urine and urinary excretion of metals in Mn exposed rats. Control ~~no~~ EDTA treatment subsequent to Mn exposure; EDTA = CaNa_2 EDTA treatment (50 mg/kg/day) subsequent to Mn exposure. Values represent means \pm S.E. of 47 determinations, treatment days only (original $n = 50$, but 3 samples/group were lost).

Figure 5.2

Urinary excretion of Mn in rats exposed chronically to Mn. (●) EDTA treated; (○) no EDTA treatment. CaNa_2 EDTA was administered interperitoneally on days 0-4 and 7-11 inclusive. Values are means \pm S.E. of 5 rats/group/day. When no error bar is shown, S.E. is smaller than the corresponding data point.

URINARY EXCRETION OF METALS

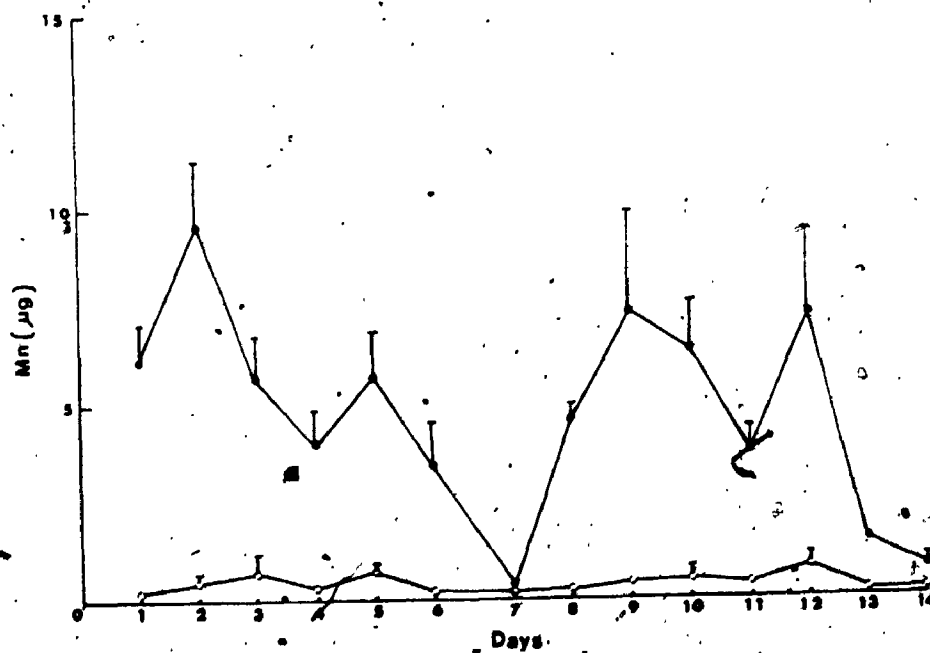
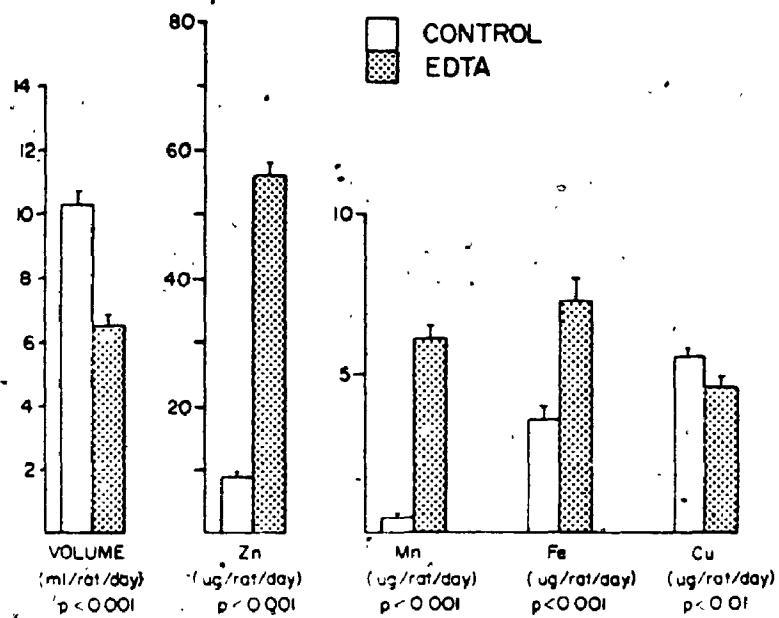
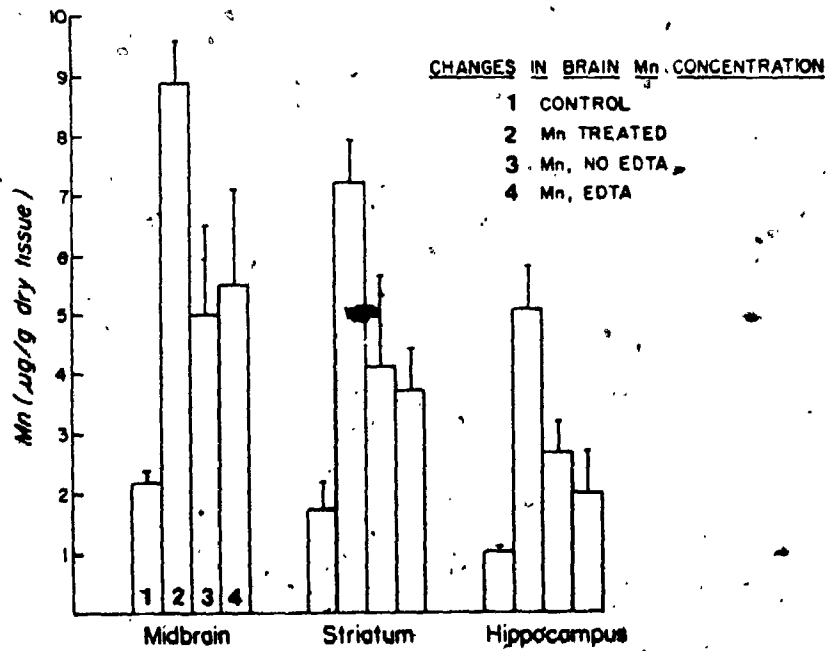
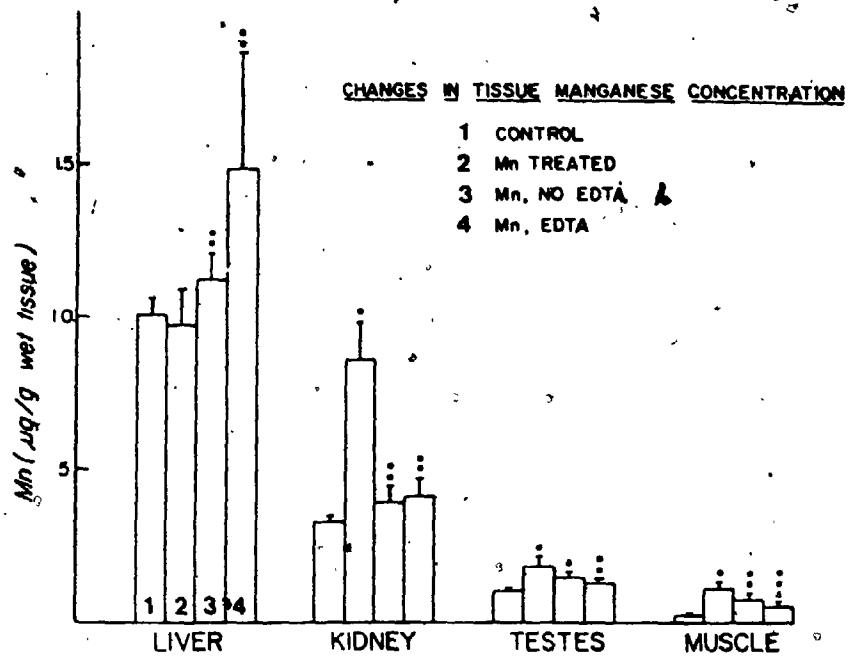


Figure 5.3

Tissue Mn concentration ($\mu\text{g/g}$ wet wt.) in unexposed rats (Group 1), rats chronically exposed to MnCl_2 for 30 days (Group 2), exposed rats for which Mn has been discontinued for two weeks (Group 3), and exposed animals subsequently treated with CaNa_2EDTA for two weeks (Group 4). Values are means \pm S.D. $n = 5$ except Group 2, $n = 6$. $p < 0.05$. Key: (\bullet) significantly different from Group 1; (\blacksquare) significantly different from Group 2; (\triangle) significantly different from Group 3:

Figure 5.4

Mn concentrations ($\mu\text{g/g}$ dry wt.) in three brain regions. Values are means \pm S.D.; $n = 5$ except for Group 2; $n = 6$. Significant differences exist in all group comparisons ($p < 0.05$) except between Groups 3 and 4 (n.s. in all cases). Midbrain = tegmentum only.



CHAPTER SIX

THE ACCUMULATION OF Mn DURING CHRONIC ORAL EXPOSURE

6.1 Methods

Two groups of 20 male Sprague-Dawley white rats (180-210 g) were used. Both groups had free access to standard rat chow (Purina Co.) and drinking water. The drinking water for the control group was distilled water containing 10 mM sucrose; that of the experimental group was the same but with the addition of 2,800 ppm Mn^{2+} as $MnCl_2 \cdot 4H_2O$. The sucrose was added to facilitate drinking and to mask any possible adverse taste due to the high level of Mn.

Rats were weighed every 20 days and the amount of water consumed was monitored. Four rats/group were killed by decapitation at 40, 80, 120, 160, and 200 days subsequent to the commencement of the study. Brains were removed immediately and placed on dry ice. Other tissues (liver, kidney, testis, pancreas, and bone) were sampled and processed for histological examination as described for the pancreas in section 4.1.5. Separate samples were prepared for metal analysis by AAS as described in section 4.1.3. Brains were dissected and processed for the determination of dopamine-receptor density and biogenic amine levels as detailed in section 4.1.6.

6.2 Results

There were no apparent differences between the control and Mn-treated groups with respect to the average amount of water consumed (Fig. 6.1), overall growth rate (Fig. 6.2), nor the growth rate of individual organs (kidney) (Fig. 6.3) over time.

The accumulation of Mn in several tissues is depicted in Fig. 6.4. Some tissues from the control group revealed a tendency toward increasing Mn concentration over the course of the study. This was especially noticeable in liver and kidney. All tissues from the treated group, however, accumulated higher levels of Mn than their corresponding controls due to treatment.

By calculating the average difference in Mn concentration between the control and treated groups for each of the sacrifice times (40, 80, 120, 160, 200 days), then plotting these differences vs. time, and finally performing regression analysis on the data, one can derive an estimate of the rate of accumulation of excess Mn/day/g tissue (i.e., the slope of the regression line). The results of these manipulations are shown in Figs. 6.5 and 6.6. When the increase in Mn concentration was expressed in absolute terms ($\mu\text{g Mn/g dry tissue}$), then the rank order of tissues with respect to the rate of accumulation of excess Mn was liver > pancreas > kidney > corpus striatum > testes > bone (Fig. 6.5). Bone and testes, in fact, revealed decreasing differences between the control and treated groups from 40 to 200 days with respect to Mn concentration indicating that the Mn content of these tissues had already reached a peak within the first 40 days of exposure.

When the differences in Mn concentration were expressed as a percent of the corresponding control concentration, the rank order of tissues with respect to the rate of accumulation of excess Mn changed to become liver > corpus striatum > pancreas > kidney > testes > bone (Fig. 6.6). This indicates that the striatum, although accumulating Mn relatively slowly in terms of absolute amounts (μg) of metal, has a relatively rapid rate of accumulation in terms of the time required to show a doubling of the normal concentration.

No significant changes in the levels of striatal DA, DOPAC, HVA, NE, 5-HT, or 5-HIAA were observed due to the oral exposure to Mn^{2+} over the course of the experiment. DA, NE, 5-HT, and 5-HIAA were also measured in the pons-medulla at 80 and 160 days, and no differences were observed between the control and treated group. The density of striatal D2-dopamine receptors was not altered due to Mn exposure. Histological examination of liver, kidney, pancreas, and testes after 200 days of exposure revealed no significant changes due to treatment.

6.3 Discussion

Even though rats in the experimental group were exposed to very high levels of Mn (the calculated daily oral dose was ~ 200 mg Mn/kg), the accumulation of excesses of the metal in tissues was very slow. Since the absorption of orally ingested Mn in rats is approximately 4% (Greenberg et al., 1963) irrespective of the dose presented (Britton and Cotzias, 1966), the Mn-treated group in the present study should have been absorbing 1-5 mg Mn/day over the course of the experiment. Thus, those rats which were sacrificed at 200 days should have absorbed a total of 200-1,000 mg Mn. However, increases in the Mn levels in all tissues examined were modest (in the μg range). Thus, it must be concluded that efficient excretion of Mn, probably via the bile (cf. Chapter 3), was responsible for preventing the accumulation of high levels of Mn. The results of the present study are in accord with those of Hughes et al. (1966) who found that a wide range of dietary manganese (0.1-1000 ppm), induced comparatively minor changes in hepatic Mn concentrations.

A comparison between oral exposure and ip injection (Chapter 4) with regard to the accumulation of excess Mn in tissues reveals that, typically, 2-3X more Mn (70X more in bone) was accumulated due to ip injection for 30 days than to oral exposure for 200 days. This is probably a consequence of the fact that systemically administered Mn is less efficiently cleared and excreted by the liver than Mn which enters the liver via the portal circulation (Thompson and Klaassen, 1982), which would be the case for an orally administered dose.

Other differences which were noted in the present study between the oral and ip administration of Mn^{2+} include: proportionally much higher levels of Mn in bone and pancreas as a result of ip injection; the absence of pathological changes in pancreatic tissue due to the oral administration of Mn; and significant increases through time in the Mn content of liver due to chronic oral exposure, an effect which was not seen after ip administration. The absence of high concentrations of Mn in the pancreas after chronic oral administration coupled with the absence of histological changes support the suggestion made in Chapter 4 that these effects are probably dependent upon the presence of high levels of Mn in the peritoneal cavity, a situation which is a direct consequence of ip injections but not of oral exposure.

The failure of the present investigation to detect changes in the levels of the biogenic amines or their metabolites in the brains of orally-exposed rats is, again, probably due to the relatively slow rate of accumulation of excess Mn when administered orally. Chandra and Shukla (1981) reported significant alterations in the levels of striatal DA, NE, and HVA in rats under experimental conditions similar to those of the present study; however, they used young animals (60 g body wt) and also reported a greater accumulation of Mn in the striatum than that observed in the present investigation. It is known that the retention of Mn after intestinal absorption (Cahill et al., 1980), the uptake of Mn into the brain (Mena et al., 1974), and the neurotoxicity of Mn (Chandra and Shukla, 1978) are all potentiated in young rats compared to adults. These facts, coupled with the negative results of the present

study, suggest that the use of young animals may be mandatory for the production of Mn-associated changes in the brain in response to chronic oral exposure.

In the present study, increases in the Mn concentration in the testes were extremely small. Even brain tissue accumulated more Mn than the testes. Similar results were reported by Rehhberg et al. (1981) who exposed pre-weanling rats orally to suspensions of Mn_3O_4 . In that study, the testes had the lowest accumulation of Mn in the organs examined (liver, kidney, brain, pituitary, testes, adrenals). It may be suggested from these results that the blood-testicular barrier (Fawcett et al., 1970) is more efficient than the blood-brain barrier in preventing the accumulation of excess Mn.

Of the tissues examined in this investigation, bone showed the most peculiar behavior with respect to the accumulation of Mn. Levels of Mn in the Mn-treated group were highest after 40 days of exposure and declined thereafter despite continued high concentrations of Mn in the drinking water. The reasons for this response are not known, nor is it known whether the Mn that is associated with bone is localized primarily in the bone matrix or in the marrow.

Figure 6.1

Average water consumption (ml/rat/day) over the first 25 weeks of the experiment. (●)

Control = distilled H₂O + 10 mM sucrose; (○)

Mn-treated = control + 2,800 ppm Mn²⁺.

Figure 6.2

Growth curves for control (●) and Mn-treated

(○) rats. There were no significant

differences between the control and treated

group at any time point.

Figure 6.3

Increase in whole kidney weight (left kidney)

over the course of the experiment. Control

(●); Mn-treated (○). Best-fit linear

-regression analysis:

Control, $Y = 8.4 \times 10^{-4}X + 0.31$, $r = 0.69$,

$p < 0.01$; Mn-treated, $Y = 7.9 \times 10^{-4}X + 0.30$,

$r = 0.74$, $p < 0.01$.

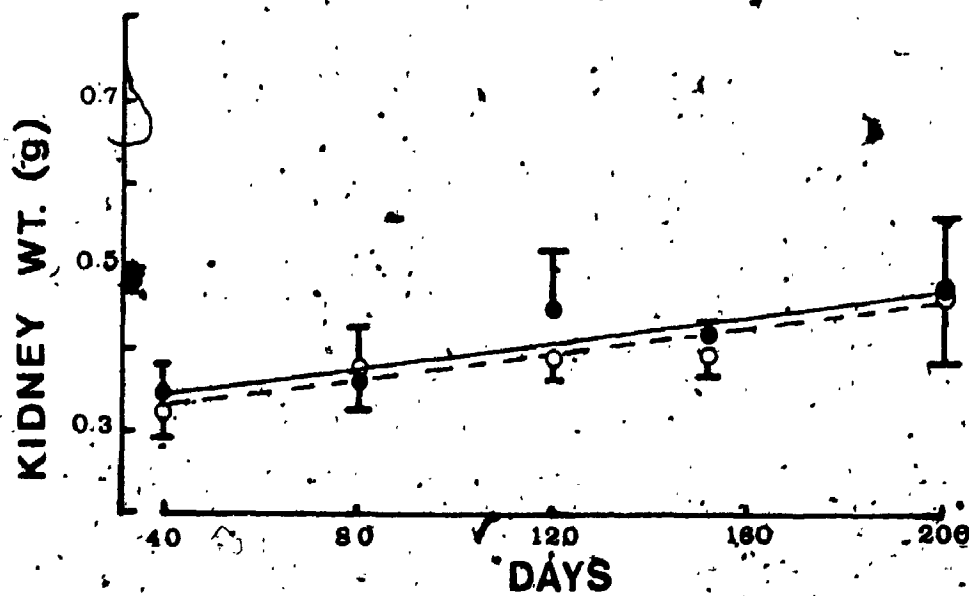
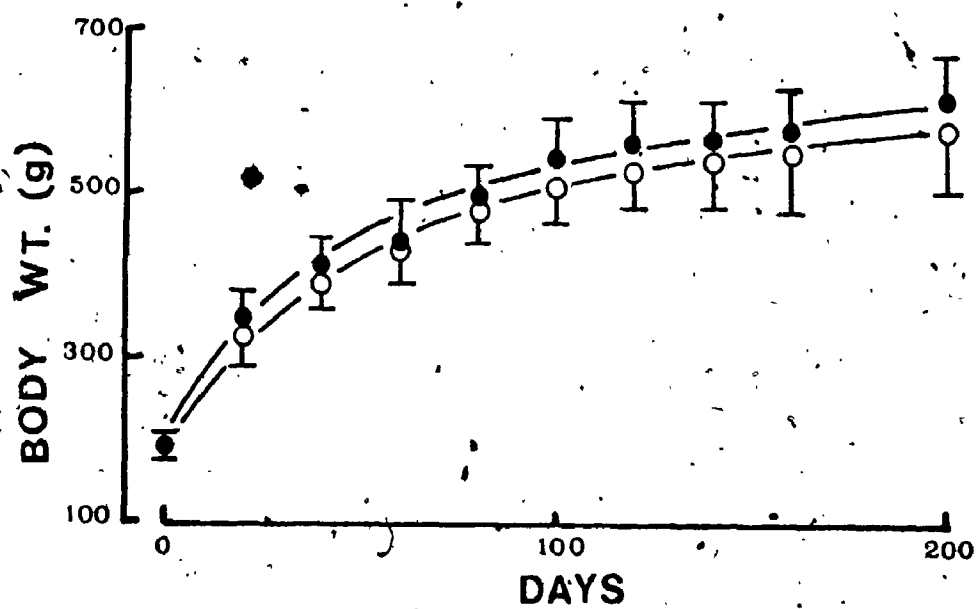
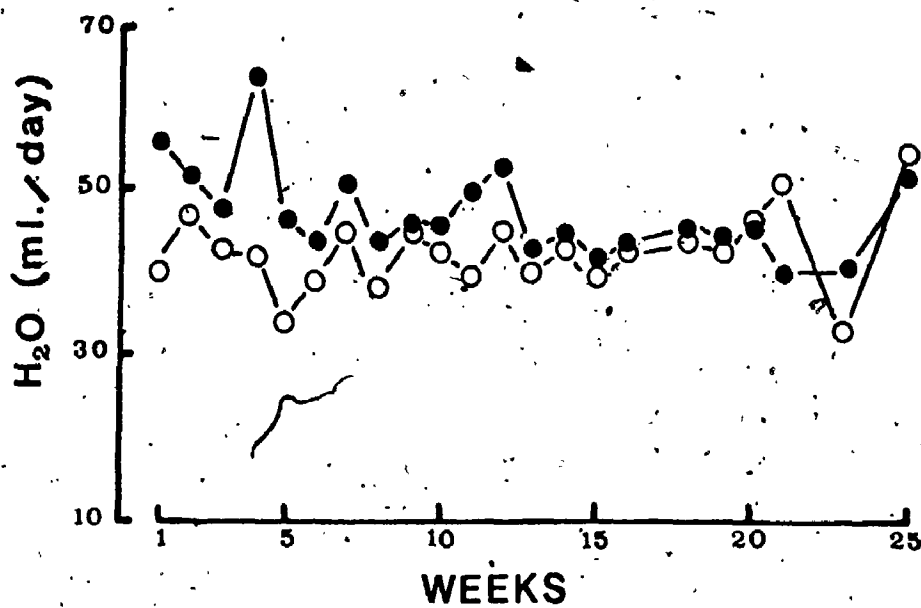


Figure 6.4

Changes in the Mn concentration of several
tissues over time. (●) Control; (○)
Mn-treated.

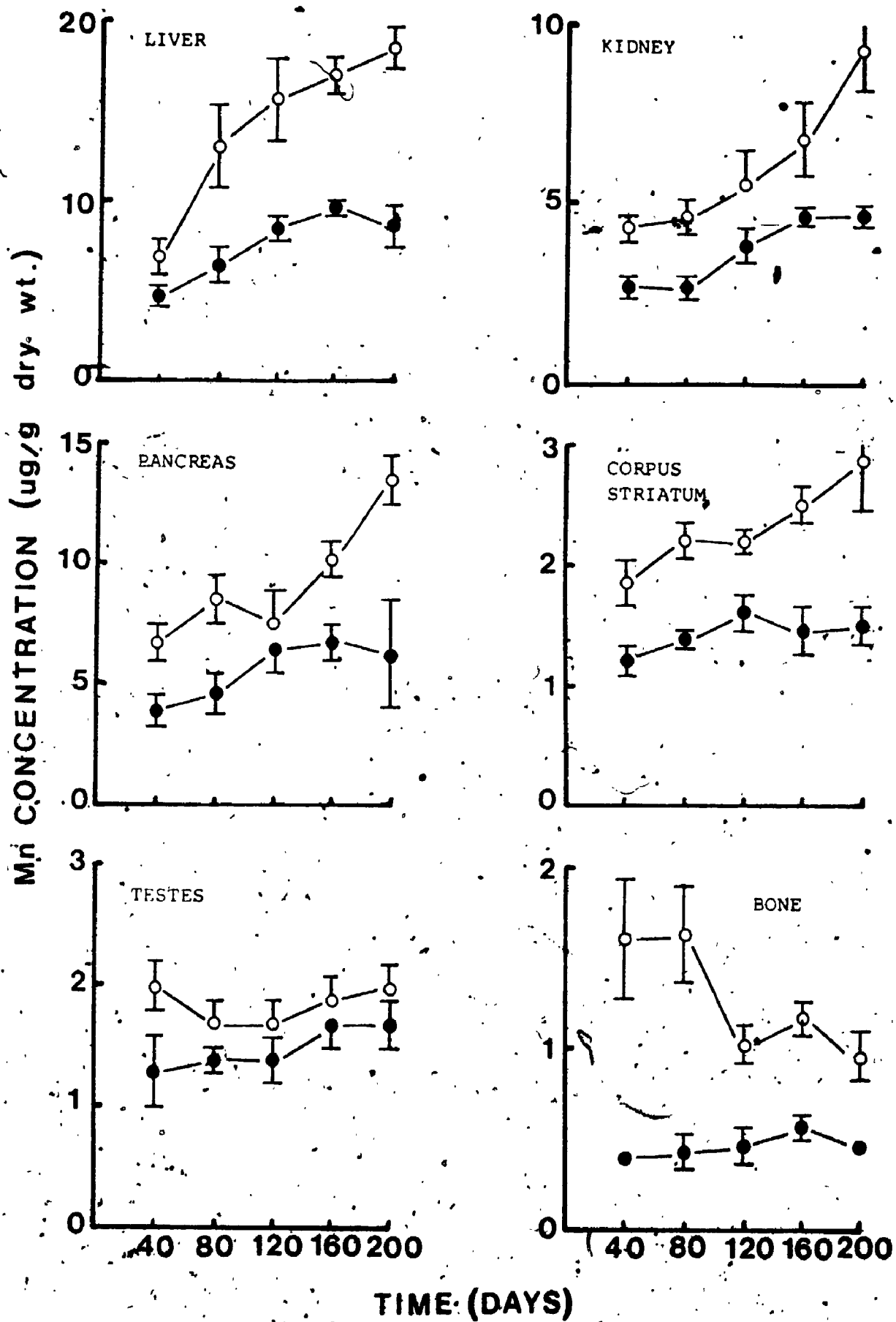


Figure 6.5

The increase over corresponding control values in the Mn concentration in tissues of the Mn-treated group. The equations of the regression lines are:

Liver: $Y = 0.042X + 1.48$

Pancreas: $Y = 0.022X + 1.07$

Kidney: $Y = 0.013X + 0.96$

Striatum: $Y = 0.004X + 0.41$

Testes: $Y = -0.002X + 0.64$

Bone: $Y = -0.005X + 1.40$

The slopes of the regression lines indicate the average daily accumulation of Mn in each tissue ($\mu\text{g Mn/g dry tissue/day}$) which is in excess of the control level from day 40 to day 200.

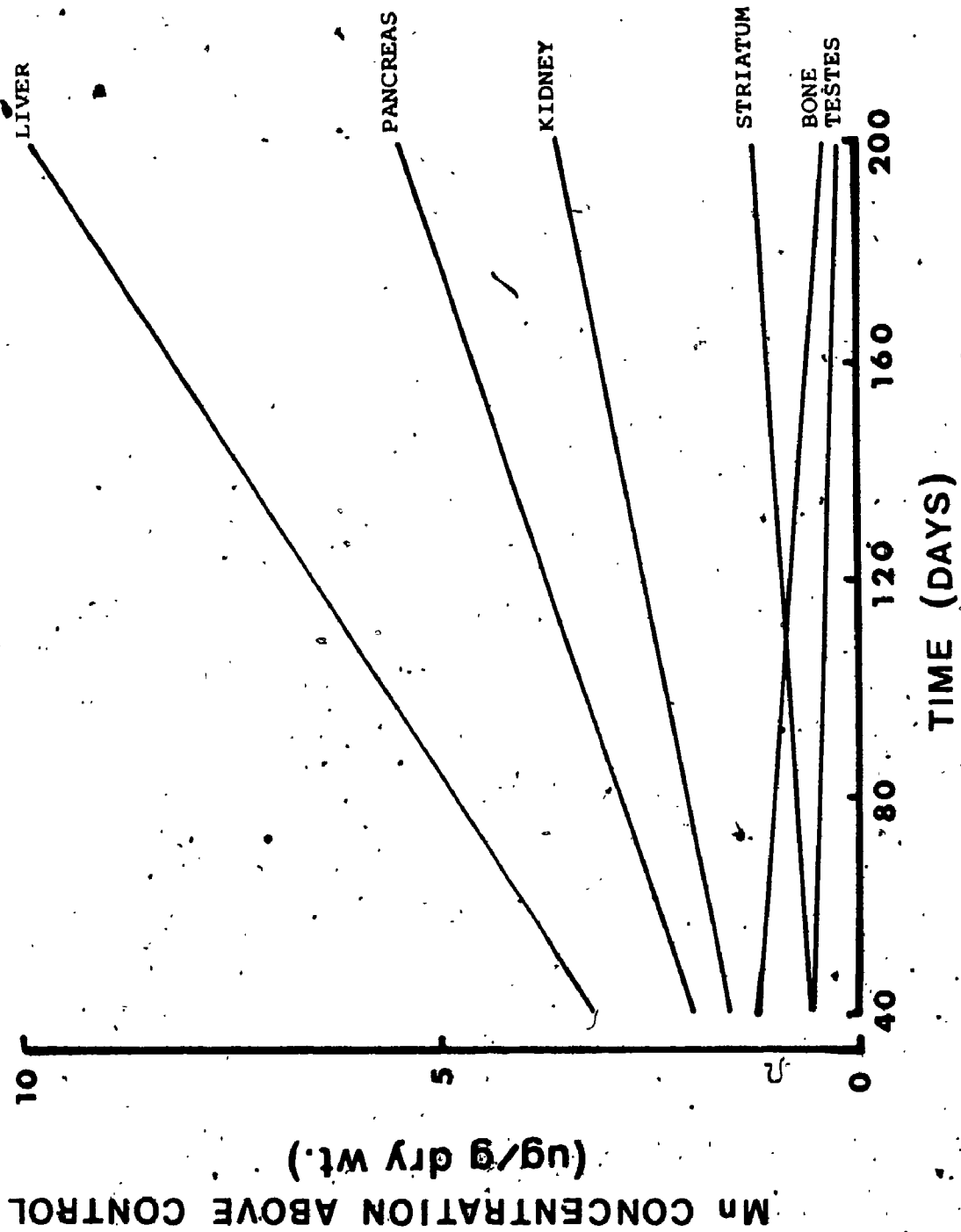


Figure 6.6

The increase over corresponding control values in the Mn concentration in tissues of the Mn-treated group. Increases are expressed as percentages of the control levels. The equations of the regression lines are:

Liver: $Y = 3.1 \times 10^{-3}X + 45$

Striatum: $Y = 2.3 \times 10^{-3}X + 37$

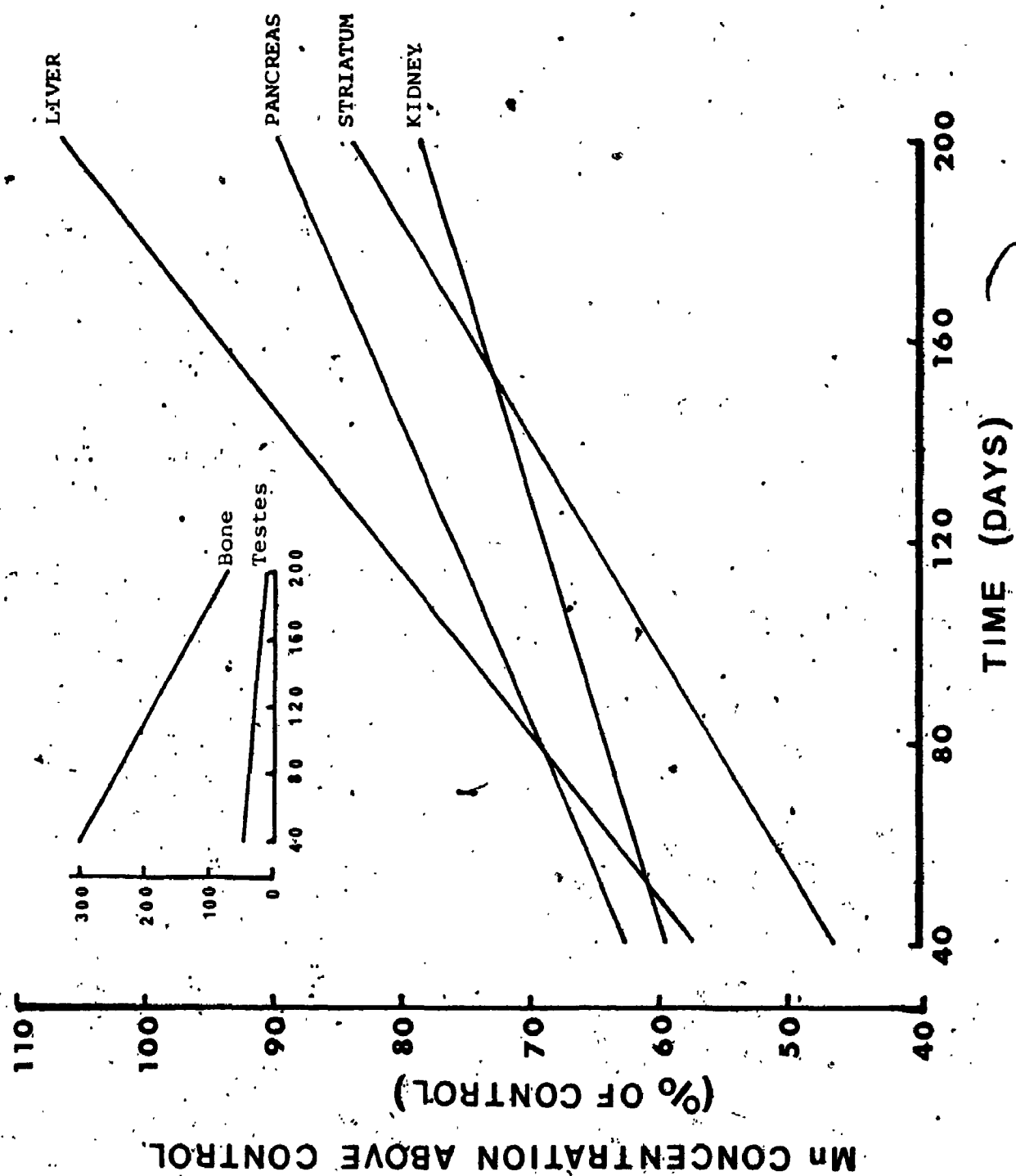
Pancreas: $Y = 1.7 \times 10^{-3}X + 56$

Kidney: $Y = 1.2 \times 10^{-3}X + 55$

Testes: $Y = -2.1 \times 10^{-3}X + 56$

Bone: $Y = -14.1 \times 10^{-3}X + 36$

The slopes of the regression lines indicate the average daily percentage increase in the Mn concentration due to treatment from day 40 to day 200.



CHAPTER SEVEN

EFFECTS OF HEAVY METAL CATIONS, SULPHYDRYL REAGENTS AND OTHER CHEMICAL AGENTS ON STRIATAL D₂-DOPAMINE RECEPTORS

In order to complement the in vivo studies of Chapters 4 and 6 in which, among other things, the effects of Mn on central dopaminergic parameters were investigated, a series of in vitro studies was planned. Initially, these experiments were designed simply to assess the effects of Mn^{2+} and other divalent metal cations on D₂ DA-receptor density when these metals were added to striatal homogenates. However, as interesting results were accumulated, the scope of the studies was broadened to include investigations aimed at elucidating some of the structural requirements of DA receptors for the specific binding of tritiated dopamine antagonists such as [³H]-spiperone (³H-spiroperidol). Heavy metal cations and a variety of biochemical agents were used as probes in these studies, which are presented here in full.

The D₂ dopamine receptor (Seeman, 1982) is characterized pharmacologically by its nanomolar affinity for neuroleptic antagonists such as the phenothiazines, thioxanthenes and butyrophenones. In contrast to antagonist binding, agonist binding to the D₂ receptor can be resolved into a high (nanomolar) affinity (D₂^{high}) and a low (micromolar) affinity (D₂^{low}) component. Guanine nucleotides are able to convert most, if not all of the D₂^{high} sites into D₂^{low} sites (Sibley et al., 1982; Wreggett et al., 1982). Antagonists are unable to resolve these two sites. Recent advances in D₂ receptor biochemistry and

pharmacology include its solubilization and partial purification (Gorissen and Laduron, 1979; Clement-Cormier and Kendrick, 1981; Lilly et al., 1982).

An aspect of the D2 receptor which has not yet been well characterized is the biochemical nature of the specific binding site. In the present study, heavy metal cations, sulphhydryl reagents, and other chemical agents were used as probes to investigate the biochemical properties of the membrane bound D2 receptor molecule with regards to specific [^3H]-spiroperidol antagonist binding. In addition, these investigations may bear relevance to the neurotoxicology of certain agents, such as manganese, in which the involvement of central dopaminergic parameters has been implicated (Mena et al., 1967; Neff et al., 1969).

7.1 Methods

7.1.1 Rat Striatal Membrane Preparation and Treatment

Sprague-Dawley rats (150-250 g) were killed by exsanguination through the abdominal aorta while under light pentobarbitol anesthesia. The brain was removed immediately and placed on dry ice, and then stored at -80°C until use. Immediately before commencement of individual experiments, striatal tissue from a sufficient number of brains was dissected over dry ice, pooled, weighed and homogenized in 30 volumes of cold MEM (ascorbate-free)¹, pH 7.4 (composition given in Table 7.1). Metals and other compounds were added to aliquots of homogenate from

stock solutions made up fresh in 20mM $\text{NaC}_2\text{H}_3\text{O}_2$ and adjusted to pH 7.4. Cu^{2+} and Fe^{2+} , however, have extremely low solubilities in the physiological pH range, therefore stock solutions of these metal cations were prepared fresh using 50-100mM Tris buffers. This system allows for the loose chelation of the metals by Tris with sufficient excess buffer to maintain the pH at 7.4. Homogenates with added reagents were incubated for various time periods at 37°C. Incubations were terminated by centrifugation at 20,000x g for 20 min. in a refrigerated centrifuge. The supernatant was discarded and the pellet resuspended in assay buffer, which was 15mM Tris/5mM EDTA, pH 7.4 unless otherwise stated.

7.1.2 Radioligand Binding Assays

D2 receptor densities were determined by quadruplicate incubations of 2-4 mg tissue for 20 min. at 20°C in 0.6 ml of 15mM Tris/5mM EDTA, pH 7.4, in the presence of 0.5nM [^3H]-SPIRO and 1 μM (+) or (-) butaclamol. Incubation was terminated by rapid filtration over Whatman GF/B glass fiber filters under vacuum. Filters were then rinsed 3 times with 5.0 ml cold Tris buffer, and radioactivity trapped on the filters was measured on a Packard liquid scintillation counter. Protein was measured by the modified Lowry method of Markwell et al. (1978). Zn-MT was isolated chromatographically from the supernatant fraction of hepatic homogenates from ZnSO_4 -injected rats as described previously

(Cherian, 1977; Onosaka and Cherian, 1982). The MT-II isoform was used in the present study. Concentrations of MT used were confirmed by pulse polarography (Olafson and Sim, 1979).

7.1.3 Preparation of Homogenates for Electron Microscopy

In order to study the morphological appearance of control striatal homogenates and those incubated in the presence of heavy metal cations, homogenates were incubated in MEM for 1 hr. at 37°C in the presence 3mM Mn^{2+} or Hg^{2+} , or in the absence of added metal. Incubation was terminated by centrifugation at 700x g for 10 min. The pellet was discarded and the supernatant fraction was further centrifuged at 20,000x g for 5 min. The supernatant was discarded and the pellets were fixed overnight at 4°C in 2.5% glutaraldehyde in .05M sodium cacodylate, pH 7.4. The pellets were washed in several changes of .05M cacodylate, pH 7.4, with 4% sucrose, and postfixed in buffered 1% osmium tetroxide at 4°C overnight. After several washes in distilled water, the pellets were dehydrated in graded ethanols, cleared in acetone, and embedded in Polybed resin. Thin silver-grey sections were stained in uranyl acetate, and viewed on a Philips EM 300, at 80 KV.

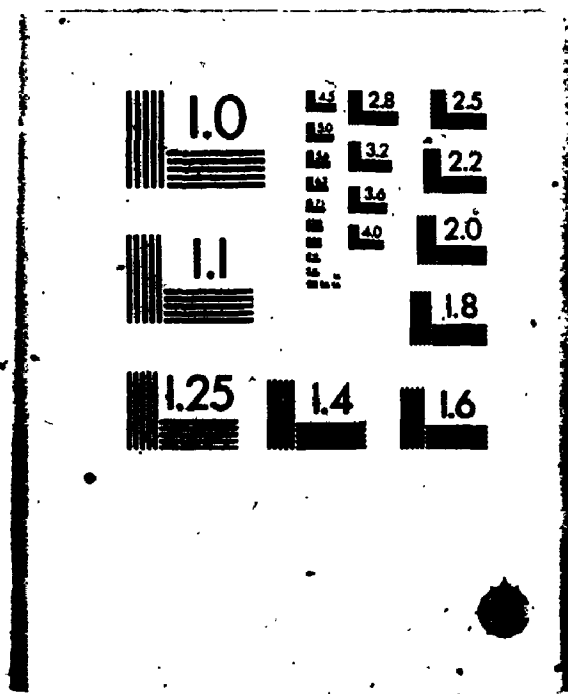
7.2 Results

7.2.1 Effects of Heavy Metals on D2 Receptor Density

Pretreatment of striatal homogenates with heavy metal cations significantly affected D2 receptor density as measured by the stereospecific binding of [^3H]-SPIRO (Table 7.2). Mn^{2+} , Fe^{2+} and Co^{2+} at 3mM concentration all enhanced subsequently measured specific binding of [^3H]-SPIRO with Mn^{2+} exhibiting the most pronounced effect. Estimation of the Mn content of striatal membranes from control and Mn^{2+} treated homogenates by atomic absorption spectrophotometry revealed that much of the exogenous Mn^{2+} had become membrane bound after incubation (Table 7.3). Scatchard analysis revealed that the influence of Mn^{2+} was to increase the maximum number of binding sites compared to control levels, while the affinity of [^3H]-SPIRO for the receptor was unchanged (Fig. 7.1). Other metals, particularly those with high affinity for sulphhydryl groups such as Cd^{2+} , Cu^{2+} and Hg^{2+} , caused a decrease in receptor sites. Pretreatment with 3mM Cd^{2+} or Cu^{2+} resulted in a 40-60% reduction in the subsequently measured stereospecific binding of [^3H]-SPIRO, while 3mM Hg^{2+} completely abolished specific [^3H]-SPIRO binding. The effect of monovalent Hg in the form of CH_3Hg^+ was similar to that of the inorganic divalent form. In addition, all metals had the common effect of increasing the nonspecific binding of [^3H]-SPIRO compared to control.

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7.2.2 Concentration Effects of Mn^{2+} and Hg^{2+}

Since Mn^{2+} and Hg^{2+} showed the most pronounced effects of the metals tested in the preliminary investigation, they were chosen for further study. As shown in Fig. 7.2, the destructive effect of Hg^{2+} was maximal between 10^{-4} and 10^{-3} M, as was the enhancing effect of Mn^{2+} . Increasing the concentration of Mn^{2+} above 10^{-3} M caused no further enhancement of specific [3H]-SPIRO binding.

7.2.3 Time Course of the Effects of Mn^{2+} and Hg^{2+}

In the absence of added metals, the incubation of striatal homogenates at $37^{\circ}C$ caused a rapid disappearance of 10-20% of D2 receptor sites, after which loss of the remaining sites was more gradual with an extrapolated $1/2$ time of about 14 hr. (Fig. 7.3). The addition of 3mM Mn^{2+} not only protected receptors from the rapid short-term loss, but it also resulted in significantly higher initial receptor densities as measured by [3H]-SPIRO. However, in order to observe the enhancing effect of Mn^{2+} , it was essential to add the metal before the incubation was begun. The addition of Mn^{2+} to striatal homogenates which had already undergone $1/2$ hr. of incubation at $37^{\circ}C$ was not effective in increasing the subsequently measured specific binding of [3H]-SPIRO compared to control (Table 7.4). The addition of 3mM Hg^{2+} caused a complete and virtually instantaneous elimination of specific [3H]-SPIRO binding sites.

7.2.4 Effects of EDTA, Ascorbate, $\text{Na}_2\text{S}_2\text{O}_5$ and NEM

Preincubation of striatal homogenates in the presence of EDTA or ascorbate enhanced D2 receptor density compared to control (Table 7.5). The effect was of a similar magnitude to that seen with Mn^{2+} , and, as illustrated in Fig. 7.4, incubation in the presence of two "enhancers" (Mn^{2+} and ascorbate) resulted in an increase which was no greater than the maximum effect produced by one agent alone. Although ascorbate produced significant increases in receptor density, incubation of homogenates with another reducing agent, $\text{Na}_2\text{S}_2\text{O}_5$, had no effect on subsequently measured D2 receptor density. The -SH alkylating agent, NEM, produced results similar to those of Hg^{2+} . Treatment of homogenates with 3mM NEM completely abolished the subsequently measured specific binding of [^3H]-SPIRO.

7.2.5 Recovery of Specific [^3H]-SPIRO Binding After Hg^{2+}

Various manipulations and additions were attempted in order to recover specific [^3H]-SPIRO binding sites subsequent to their elimination by Hg^{2+} . It was reasoned that if Hg^{2+} could be removed from the membrane, then some or all of the lost binding sites might be regenerated. However, washing the membranes with Tris/EDTA or Tris/DMPS buffers did not result in the recovery of any lost binding sites, nor did the incubation of Hg^{2+} -treated membranes with excess EDTA or DMPS

for an additional 1/2 hr. (Fig. 7.5). A similar incubation in the presence of 3mM DTE, however, was effective in regenerating approximately 40% of lost binding sites.

7.2.6 Protection of D2 Receptors from Destruction by Hg^{2+}

Generally, agents which tended to enhance specific [^3H]-SPIRO binding to striatal membranes, such as Mn^{2+} and EDTA, were unable to protect receptors from the destructive effect of Hg^{2+} when added to striatal homogenates prior to Hg^{2+} (Fig. 7.5). Ascorbate protected slightly, but this was due to its ability to remove Hg^{2+} from the system by reducing Hg^{2+} to Hg^+ which was promptly precipitated as dark grey Hg_2O or HgOH . The chelating agent DMPS (5mM), a water soluble derivative of BAL with high affinity for Hg^{2+} , was unable to protect receptors against 1mM Hg^{2+} , but the disulphide-bridge reducing agent DTE (3mM), although it caused a 30% reduction in specific [^3H]-SPIRO binding sites when added alone, afforded complete protection to receptors when added to homogenates immediately prior to Hg^{2+} . Addition of the Hg-binding protein metallothionein to homogenates in sufficient concentration to theoretically complex all of the subsequently added Hg^{2+} failed to protect against the effect of the metal, and in fact itself caused a reduction of about 50% of the specific [^3H]-SPIRO binding sites (Table 7.6). By reducing the concentration of Zn-MT to a level which had no independent effect on D2 receptor density, a significant protection of 25-30% of sites was observed after the

addition of 1mM Hg^{2+} . Formation of the SPIRO/receptor complex prior to the addition of Hg^{2+} also tended to protect specific binding sites from destruction by Hg^{2+} . As depicted in Fig. 7.6, the normal dissociation rate of $[^3\text{H}]\text{-SPIRO}$ in the absence of added metal was not rapid under the conditions used in the present study. The extrapolated $1/2$ time of the $[^3\text{H}]\text{-SPIRO/receptor}$ complex was 1.5 hr. after the initial 20 min. incubation. 1mM Hg^{2+} , a concentration which completely abolished specific binding when added to crude striatal homogenates, when added to membrane suspensions after the standard 20 min. $[^3\text{H}]\text{-SPIRO}$ binding incubation caused a rapid dissociation of 20% of specifically bound $[^3\text{H}]\text{-SPIRO}$, within 5 min. of the addition of Hg^{2+} , but thereafter the dissociation rate was similar to the control rate. The effect of 3mM Hg^{2+} was more dramatic causing a loss of 90% of specific $[^3\text{H}]\text{-SPIRO}$ binding within 10 min. of the addition of the metal.

7.2.7 Morphological Appearance of Striatal Membranes

Transmission electron microscopy of the 20,000x g pellet from control, Hg^{2+} treated and Mn^{2+} treated striatal homogenates revealed significant differences among the groups (Fig. 7.7, Table 7.7). Controls were characterized by large numbers of vesicular structures per field. Synaptosomes were frequently observed. In stark contrast, Hg^{2+} -treated membranes were characterized by a much lower density of vesicles, most of which were devoid of contents. Rather than being organized into vesicular structures, membranes from Hg^{2+} -treated homogenates were

fragmented and the fields were strewn with debris. Intact synaptosomes were never observed. Mn^{2+} -treated homogenates, on the other hand, retained a morphological appearance similar to that of the control.

7.3 Discussion

A major finding of the present study is that preincubation of striatal homogenates in the presence of millimolar Mn^{2+} , EDTA, or ascorbate results in an enhancement of the subsequently measured stereospecific binding of [3H]-SPIRO. The simplest explanation for this effect is to hypothesize that these agents protect receptor sites from degradation during the preincubation period. Indeed, Fig. 7.3 demonstrates the ability of 3mM Mn^{2+} to inhibit the rapid short-term loss of specific [3H]-SPIRO binding sites which is observed in control homogenates incubated in the absence of added Mn^{2+} . This loss of sites may be due to the release of proteolytic enzymes and/or to lipid peroxidation in the membranes (Heikilla and Cabbat, 1983). Mn^{2+} , if added to homogenates subsequent to a period of preincubation in the absence of Mn^{2+} , is unable to induce recovery of these lost sites. However, as both Fig. 7.3 and 7.4 indicate, the addition of 3mM Mn^{2+} and/or 1mM ascorbate to striatal homogenates also results in a significantly increased density of [3H]-SPIRO binding sites even before any incubation has taken place. Thus, it may be that Mn^{2+} somehow causes the unmasking of a subpopulation of receptors which would otherwise go undetected, or, alternatively, Mn^{2+} (or EDTA or ascorbate) may enhance the recovery of

specific binding sites which are lost in the absence of these agents. Evidence for this latter possibility is presented in Table 7.8. It was found that the 20,000x g pellet from aliquots of homogenate incubated in the presence of Mn^{2+} , when resuspended in buffer and analysed for protein content contained approximately 130% of the protein measured in controls. Hg^{2+} treatment resulted in a similar elevation in recovered protein (data not shown), as did $(NH_4)_2SO_4$ precipitation. The concurrent enhancement of $[^3H]$ -SPIRO binding sites observed in Mn^{2+} -treated homogenates was of an almost identical magnitude ($\sim 130\%$) in the absence of any preincubation, and even greater when homogenates were incubated at $37^\circ C$ prior to the measurement of specific $[^3H]$ -SPIRO binding. Treatment of homogenates with $(NH_4)_2SO_4$, while enhancing protein recovery, caused a 40-50% reduction in specific $[^3H]$ -SPIRO binding sites (data not shown). Thus, agents which increase the apparent D2 receptor density in striatal membranes probably do so by a process of protection against receptor degradation combined with an enhancement in the recovery of specific binding sites during centrifugation. The exact mechanisms by which these effects occur are not known. It was thought that the production of these effects might be a general property of antioxidants, but incubation of striatal homogenates in the presence of the reducing agent $Na_2S_2O_5$ did not result in any enhancement of the subsequently measured D2 receptor density (Table 7.5). However, regarding the protection of receptors, it may be significant to note that both enzymatic and non-enzymatic lipid peroxidation occurs in brain synaptosomal and microsomal preparations incubated at $37^\circ C$ (Baishayee and Balasubramanian, 1971), and that at appropriate concentrations, Mn^{2+} , EDTA and ascorbate all inhibit lipid

peroxidation in membrane suspensions (Barber, 1963; Bishayee and Balasubramanian, 1971). Lipid peroxidation has been implicated as an important factor causing the unwanted reduction of specific ^3H -ligand binding to D2 receptors (Chan et al., 1982; Heikkilä and Cabbat, 1983). Regarding the ability of Mn^{2+} to enhance the recovery of receptors in the absence of any preincubation of homogenates, it should be noted that the microsomal fraction from striatal homogenates, which would not normally be expected to sediment at 20,000x g, is enriched with specific [^3H]-SPIRO binding sites (Leysen and Laduron, 1977; Laduron et al., 1978). Thus, the addition of Mn^{2+} (or EDTA or ascorbate) may cause sedimentation of subcellular components not normally recovered at 20,000x g resulting in enhanced recovery of both total protein and specific [^3H]-SPIRO binding sites. This effect may also explain the enhancement of receptor density caused by Fe^{2+} and Co^{2+} , and the fact that heavy metals in general cause increases in the non-specific binding of [^3H]-SPIRO (Table 7.2).

A second major finding of the present series of investigations is that the incubation of striatal homogenates in the presence of sulphydryl reagents including the sulphydryl-reactive metal cations Zn^{2+} , Cd^{2+} , Cu^{2+} , Hg^{2+} and CH_3Hg^+ , as well as the sulphydryl-alkylating agent NEM, cause a variable loss of D2 receptors as measured by subsequent [^3H]-SPIRO binding assays. The degree of receptor loss caused by heavy metal cations ($\text{Hg}^{2+} > \text{Cu}^{2+} > \text{Cd}^{2+} > \text{Zn}^{2+}$) is roughly correlated with their affinity for sulphydryl groups. The results obtained in the present study are in accord with those of Aronstam et al. (1978) who found a similar order of potencies for heavy metal inhibition of

specific [^3H]-QNB binding to cerebral muscarinic receptors. Metals with lower sulphydryl reactivity such as Mn^{2+} , Co^{2+} , Fe^{2+} , Ni^{2+} and Sn^{2+} were unable to inhibit specific [^3H]-QNB binding.

The destructive effect of Hg^{2+} on the membrane-bound D2 receptor is immediate upon the addition of the metal, and the effect cannot be antagonized by the prior addition of substances which, by themselves, cause an enhancement of D2 receptor density such as Mn^{2+} or EDTA. Similarly, Mn^{2+} was unable to protect specific [^3H]-SPIRO sites from destruction by NEM. These results indicate that the detrimental effects of Hg^{2+} and NEM are not simply due to a stimulation of the natural degradation rate of the receptors. Rather, it is likely that Hg^{2+} and NEM bind to one or more vital -SH groups located on or in close proximity to the receptor site and that this results in a conformational change which prevents specific [^3H]-SPIRO binding. Prior formation of the SPIRO/receptor complex renders these -SH groups less accessible to Hg^{2+} . Likewise, prior addition of DTE to striatal homogenates protects these essential -SH groups from occlusion by Hg^{2+} . DMPS and Zn-MT, however, were ineffective in protecting specific [^3H]-SPIRO sites from destruction by Hg^{2+} . This was surprising since DMPS, unlike EDTA, has a high affinity for Hg^{2+} , and has been found to be an effective in vivo chelating agent for both inorganic and organic forms of Hg (Gabard, 1976a; Gabard, 1976b). Similarly, MT is a low molecular weight (6,500) cysteine-rich protein with very high affinity for Hg^{2+} (Kagi and Vallee, 1961; Piotrowski et al., 1973). Zn bound to MT is readily displaced by Hg^{2+} . It was therefore predicted that DMPS and Zn-MT would be able to protect all or most receptors from destruction by Hg^{2+} . The fact that these agents were unable to do so indicates that the simple

ability to complex Hg^{2+} is not a sufficient condition to enable a substance to protect receptors from occlusion by Hg^{2+} . Further studies are required to determine more precisely which biochemical properties are important in protecting D2 receptors from damage by sulphhydryl reagents.

The high affinity agonist conformation of the D2 receptor (D2^{high}) may be more sensitive to -SH reagents than the D2^{low} site. Suen et al. (1980) found that specific high-affinity [^3H]-dopamine binding to calf striatal membranes was reduced to 40% of the control level in the presence of 0.1 mM NEM, a concentration which had no effect on specific [^3H]-SPIRO binding. The fact that specific ^3H -antagonist binding remained unaffected indicates that 0.1mM NEM may cause conversion of receptors from a D2^{high} to a D2^{low} configuration. Guanine nucleotides have been shown to convert D2^{high} to D2^{low} sites (Sibley et al., 1982). Reportedly, NEM exerts an opposite effect on [^3H]-agonist binding to muscarinic receptors, converting receptors from a state of low agonist affinity to high agonist affinity (Aronstam et al., 1978). A complicating factor in the Suen et al. study is that under the agonist assay conditions used in that report (40nM [^3H]-dopamine in the presence or absence of 10uM (+)-butaclamol), [^3H]-dopamine will label D3 sites (Seeman, 1980) as well as D2^{high} sites. Labelling of D3 sites may comprise as much as 50% of the specific ^3H -agonist binding to striatal membranes (Leff and Creese, 1983), thus it becomes difficult to clearly interpret the effect of NEM on the D2 agonist site. In the present study, pretreatment of striatal homogenates with 3mM NEM resulted in the complete abolition of [^3H]-antagonist ([^3H]-SPIRO) binding. This is in agreement with the results of Freedman et al. (1982) who reported that

preincubation of striatal membranes in 1mM NEM caused a reduction of subsequently measured specific [^3H]-sulpiride binding to 36% of the control level. Receptors could be protected from the effect of NEM by the prior addition of dithiothreitol. We have demonstrated here that DTE was the only agent of several which were examined that could completely inhibit the destructive effect of Hg^{2+} on D2 receptor density, and the only agent which could partially reverse the loss of specific [^3H]-SPIRO sites after treatment of homogenates with Hg^{2+} . The ability of a substance to regenerate free -SH groups may thus be a requirement to protect receptors from sulphhydryl reagents or to reverse their detrimental effects.

In Section 1.3.2.3, the ability of intrastriatal microinjections of Mn^{2+} to cause behavioral effects in rats which are similar to those caused by microinjection of neuroleptic drugs was noted (Inoue et al., 1975). It was speculated that, since neuroleptics act by blocking DA receptors, the effects of Mn^{2+} may be mediated by a similar mechanism. However, the present in vitro study, in which striatal homogenates were incubated in the presence of high concentrations of Mn^{2+} without detrimental effects on D2 receptor density, as well as the in vivo studies of Chapters 4 and 6 in which significant increases in striatal Mn concentration failed to alter the normal striatal D2-DA receptor density do not support a direct effect of Mn on DA receptors in Mn-induced neurotoxicity.

Footnotes:

- ¹ Lipid peroxidation can produce deleterious effects on membrane function in vitro (Schaefer et al., 1975). The non-enzymatic formation of lipid peroxides in tissue homogenates is catalysed by Fe^{2+} which is oxidized to Fe^{3+} in the process. When Fe^{2+} has been depleted by conversion to Fe^{3+} , the production of lipid peroxides falls off (Bernheim, 1963). However, ascorbate, over a specific range of low concentrations, selectively reduces Fe^{3+} to Fe^{2+} thus effectively regenerating the catalytic agent and causing a sustained enhancement of lipid peroxide production (Bernheim, 1963). Ascorbate-induced lipid peroxidation has been implicated as an important factor leading to the unwanted reduction of specific [^3H]-antagonist binding to D2 dopamine receptors (Chan et al., 1982; Heikilla and Cabbat, 1983). The contention that this reduced binding may be due to lipid peroxidation is supported by the finding that the dose-response curves for the ascorbate-related enhancement of lipid peroxidation as well as the ascorbate-induced destruction of [^3H]-ligand binding to receptors both display a biphasic character, and both processes are inhibited by EDTA (Barker, 1963; Leslie et al., 1980; Heikilla and Cabbat, 1983). The biphasic character is due to the fact that, at higher concentrations, ascorbate ceases to selectively reduce Fe^{3+} , but rather acts as a general antioxidant thus inhibiting lipid peroxide formation. Because of these complicating factors, ascorbate was deliberately excluded from the incubation medium and from the standard assay buffer used in the present series of investigations.

Table 7.1

Composition of MEM, pH 7.4, Used for Homogenization and
Incubation of Striatal Tissue

In addition to the major components listed below, 13 amino acids
and 8 vitamins were present in trace amounts. No ascorbate was
present.

Components	Concentration ($\mu\text{g/ml}$)
CaCl_2	200
KCl	400
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	200
NaCl	6800
NaHCO_3	2200
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	140
Glucose	1000

Table 7.2

Effects of Heavy Metal Cations on Striatal D2 Receptor Density

Equal volume aliquots of rat striatal homogenate were incubated for 1 hr. at 37°C in the presence or absence of 3mM metals. Dopamine receptor density was subsequently determined in the 20,000x g pellet using the standard [³H]-SPIRO binding assay described in Methods.

^a Percent of control

^b Mean \pm S.D., n=3

Addition	[³ H]-SPIRO Bound ^a	
	Stereospecific Binding	Non-specific Binding
None (control)	100	100
Mn ²⁺	^b 170 \pm 32	187 \pm 14
Fe ²⁺	127 \pm 18	170 \pm 17
Co ²⁺	125 \pm 6	136 \pm 18
Zn ²⁺	86 \pm 10	122 \pm 1
Cd ²⁺	53 \pm 5	145 \pm 25
Cu ²⁺	43 \pm 19	172 \pm 18
Hg ²⁺	7 \pm 6	139 \pm 12
CH ₃ Hg ⁺	15 \pm 5	115 \pm 15

Table 7.3

Mn Content of Striatal Membranes

Striatal homogenates (5 ml vol.) were incubated in the presence or absence of 3mM Mn^{2+} for 1 hr. at 37°C. Incubation was terminated by centrifugation and the 20,000x g pellet was resuspended in 15mM Tris/5mM EDTA, pH 7.4. Mn content was then measured by flameless atomic absorption spectrophotometry.

*Total Mn added to the homogenate was 0.83 mg.

Additions	Total Mn (μ g)	μ g Mn/g Wet Wt. Tissue
None (control)	0.6	6
* Mn^{2+}	452	4.4×10^3

Table 7.4

Effect of Mn^{2+} on Striatal D2 Receptor Density

Homogenates were incubated for a total of 1 hr. at 37°C. 3mM Mn^{2+} when added before commencement of incubation enhanced subsequently measured [3H]-SPIRO binding, but had no effect if added after 30 min. incubation in the absence of metal.

^a Percent of control

^b Mean \pm S.D., n=3

[3H]-SPIRO Bound ^a		
Additions	Stereospecific Binding	Non-specific Binding
None (control)	^b 100	100
Mn^{2+} (at 0 min.)	142 \pm 13	119 \pm 5
Mn^{2+} (at 30 min.)	106 \pm 7	101 \pm 5

Table 7.5

Effects of EDTA, Ascorbate, $\text{Na}_2\text{S}_3\text{O}_5$ and NEM on Striatal D2 Receptor Density

Crude striatal homogenates were incubated for 1 hr. at 37°C in the presence or absence of the indicated agents. Incubation was terminated by centrifugation and [^3H]-SPIRO binding was determined in the resuspended 20,000x g pellet.

* Mn^{2+} was added 1 min. prior to NEM

^a Percent of control

^b Mean \pm S.D., n=3

Additions	Stereospecific Binding	Non-specific Binding
None (control)	100	100
EDTA (1mM)	^b 152 \pm 25	111 \pm 5
Ascorbate (5mM)	150 \pm 16	105 \pm 4
$\text{Na}_2\text{S}_3\text{O}_5$ (3mM)	94 \pm 9	95 \pm 4
NEM (3mM)	9 \pm 13	106 \pm 15
* NEM + Mn^{2+} (3mM)	10 \pm 10	123 \pm 10

Table 7.6

Modulation by Zn-MT of Hg^{2+} -induced Loss of Striatal D2 Receptors

The metal binding protein metallothionein was added to striatal homogenates 1 min. prior to Hg^{2+} in an attempt to protect receptors from destruction by the metal. Each mole of protein binds 7 g atoms of metal when saturated.

^a Percent of control

^b Mean \pm S.D., n=3

Additions	$[^3\text{H}]$ -SPIRO Bound ^a	
	Stereospecific Binding	Non-specific Binding
None (control)	100	100
Zn-MT (~0.15mM)	^b 47 \pm 6	84 \pm 6
Zn-MT + Hg^{2+}	0.8 \pm 1.2	166 \pm 11
Zn-MT (~0.03mM)	114 \pm 14	96 \pm 8
Zn-MT + Hg^{2+}	27 \pm 4	146 \pm 26

Table 7.7

Morphometric Analysis of Striatal Membranes

The number of "vesicular bodies" from random electron photomicrographic fields of equal magnification from control, Mn^{2+} -treated and Hg^{2+} -treated striatal homogenates were counted, and the proportion of vesicular structures which appeared devoid of contents was determined. Very few vesicular structures were present in Hg^{2+} -treated membrane samples, and a high proportion were empty.

^a Mean \pm S.D., n=3.

* Significantly different from control, $p < 0.01$.

	Vesicular Bodies/Field	% Empty
Control	^a 62 \pm 11	24 \pm 3
Mn^{2+}	40 \pm 6	28 \pm 6
Hg^{2+}	* 23 \pm 5	* 66 \pm 4

Table 7.8

Protein Content of Striatal Membranes

Protein was measured in the resuspended 20,000x g pellet from equal aliquots of rat striatal homogenate incubated for 1 hr. at 37°C in the presence or absence of 3mM Mn^{2+} . A drop of saturated $(NH_4)_2SO_4$ was added to one control homogenate immediately prior to centrifugation. Incubation with Mn^{2+} had the same effect as $(NH_4)_2SO_4$ precipitation in raising the apparent protein content of striatal membranes.

^a mg/g original wet tissue wt.

^b Mean \pm S.D. of 3 replications.

	^a Protein
Control	^b 69 \pm 2
$(NH_4)_2SO_4$	87 \pm 1
Mn^{2+} (3mM)	91 \pm 6

FIGURES

Figure 7.1

Scatchard plot of stereospecific binding of [^3H]-SPIRO. Striatal membranes were preincubated for 1 hr. at 37°C in the presence or absence of 3mM Mn^{2+} . The [^3H]-SPIRO binding assay was performed under standard conditions using 1.7 mg tissue per incubation tube and five concentrations of [^3H]-SPIRO (0.03–0.50 nM). Control (●): $B_{\text{max}} = 140$ fmol/mg protein, $K_D = 0.21$ nM; Mn (■): $B_{\text{max}} = 193$ fmol/mg protein, $K_D = 0.20$ nM). B_{max} and K_D were calculated from the best-fit linear regression lines through the data points. A single-site model for the specific binding of [^3H]-SPIRO was assumed. Hill coefficients were: control, 1.14; Mn-treated, 1.07.

Figure 7.2

Effects of increasing concentrations of Hg^{2+} and Mn^{2+} . Incubations were for 1 hr. at 37°C . The 20,000 \times g pellet was washed once before a final resuspension in standard assay buffer (15 mM Tris/5 mM EDTA, pH 7.4). Effects on the stereospecific binding of [^3H]-SPIRO due to the preincubation treatments were then determined. Data points are means \pm S.D., $n=3$.

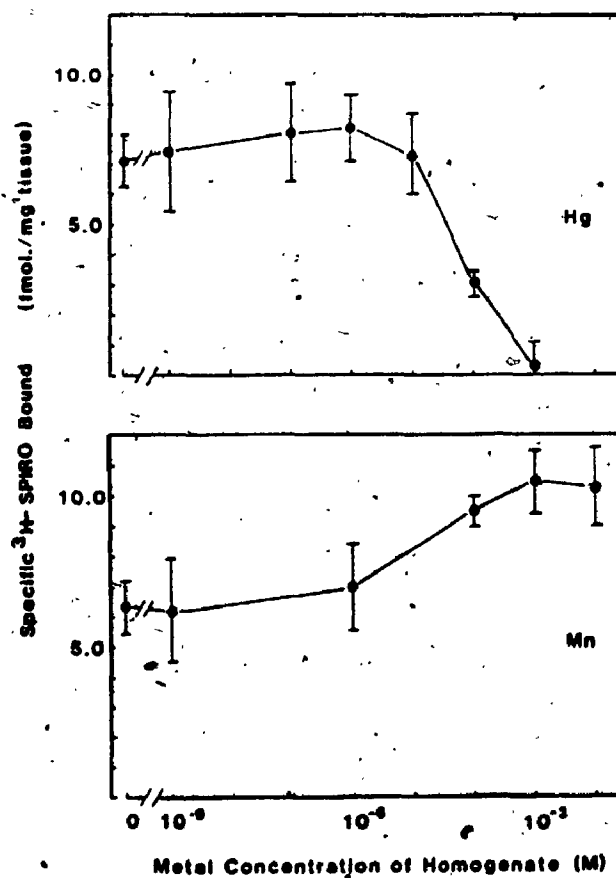
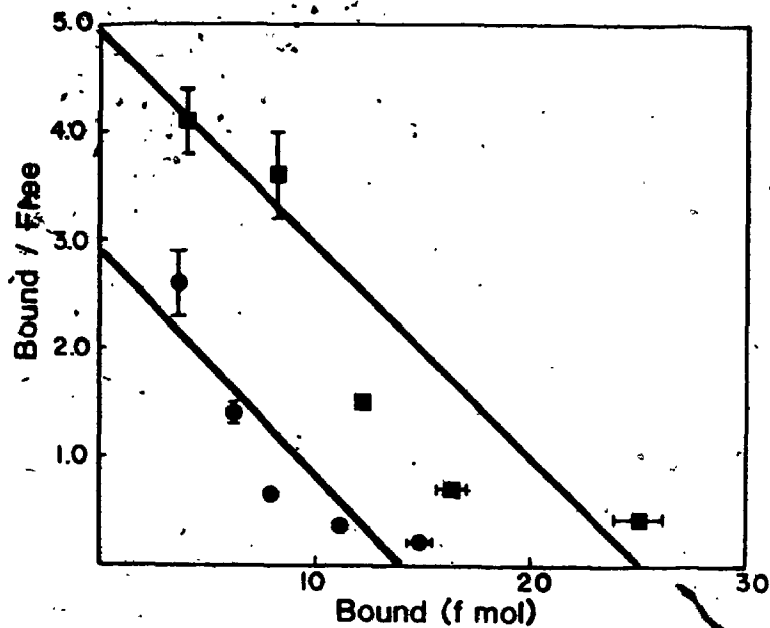


Figure 7.3

Time course of the loss of specific [^3H]-SPIRO binding sites. Striatal homogenates were preincubated in MEM for 0.25-6.0 hr. in the presence or absence of 3mM metals). Control (\bullet); Mn^{2+} (\blacktriangle); Hg^{2+} (\blacksquare). Data points are means \pm S.D., n=3.

Figure 7.4

Combined effect of Mn^{2+} and ascorbate on [^3H]-SPIRO binding. Equal volume aliquots of a striatal homogenate were either centrifuged immediately (control, no incubation), or incubated at 37°C for 1.5 hr. in the presence of no additions (control), Mn^{2+} (3mM), ascorbate (1mM), or Mn^{2+} + ascorbate. [^3H]-SPIRO binding was determined subsequent to centrifugation and resuspension of pellets in 15mM Tris/5mM EDTA, pH 7.4. Enhancement of D2 receptor density was maximal with Mn^{2+} alone.

*Stereospecific binding of [^3H]-SPIRO as a percent of non-incubated control; mean \pm S.D., n=3.

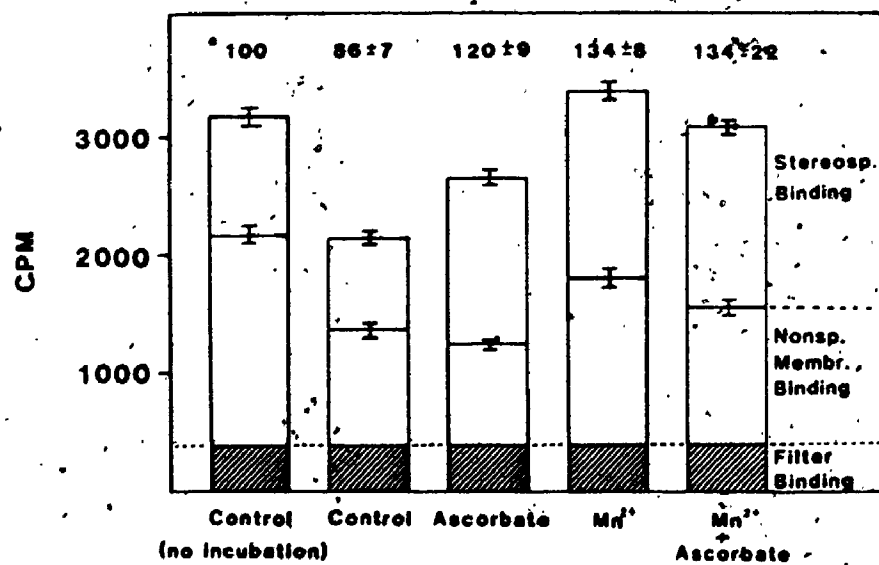
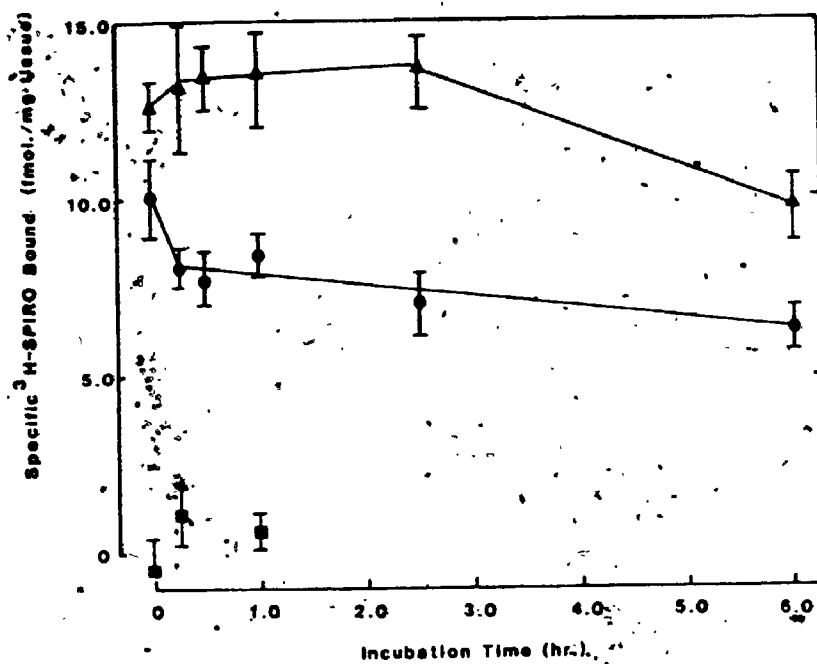


Figure 7.5

Recovery of specific [^3H]-SPIRO binding sites after pretreatment with Hg^{2+} . Equal volume aliquots of a striatal homogenate were incubated in MEM for 1/2 hr. in the presence of 1mM Hg^{2+} , then for an additional 1/2 hr. in the presence of 5mM EDTA, 5mM DMPS, or 3mM DTE. Specific [^3H]-SPIRO binding was subsequently determined under standard assay conditions. Control = incubation for 1 hr. in the absence of any additions. DTE was the only agent tested which caused a significant regeneration of specific binding sites lost due to treatment with Hg^{2+} . Data are means \pm S.D., n=3.

Figure 7.6

Protection of specific [^3H]-SPIRO binding sites from destruction by Hg^{2+} . Substances were added to crude striatal homogenates 1 min. prior to Hg^{2+} . Concentrations used were: Hg^{2+} , EDTA, 1mM; Mn^{2+} , DTE, 3mM; DMPS, ascorbate, 5mM. Incubation was for 1 hr. at 37°C. 3mM DTE protected 100% of specific binding sites from 1mM Hg^{2+} . Data are means \pm S.D., n=3.

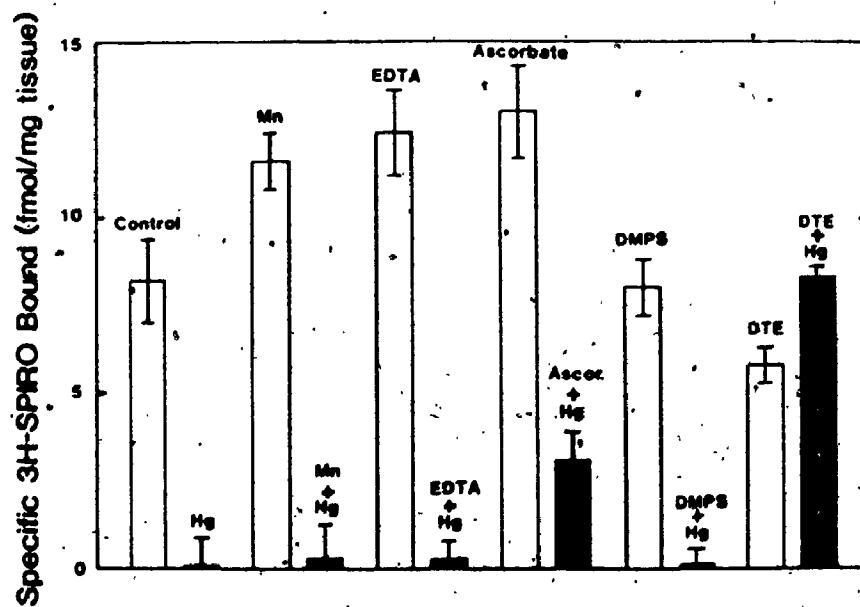
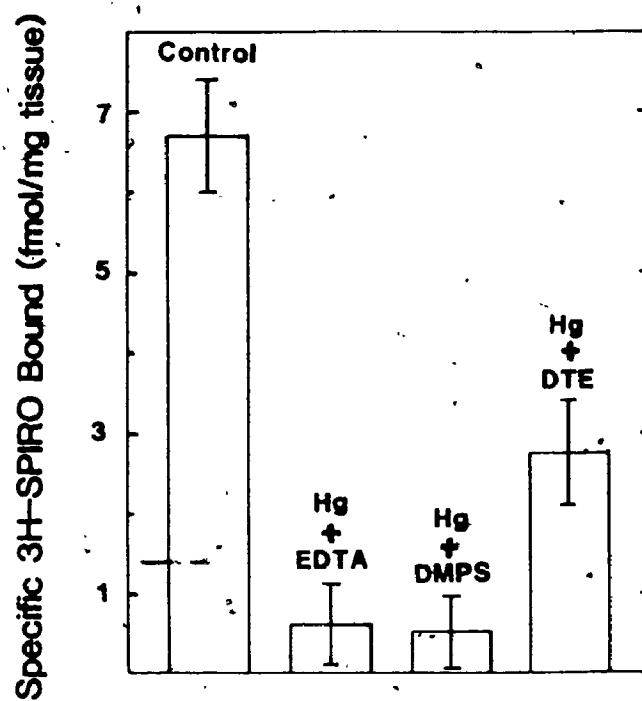


Figure 7.7

The dissociation of specific [^3H]-SPIRO bound to striatal membranes. The standard 20 min. binding assay was carried out before the addition of 0 (●), 1 (□), or 3 (■) mM Hg^{2+} and incubation was then allowed to continue for 1, 2, 5, 10 or 30 min. By extrapolation, the $t_{1/2}$ of the SPIRO/receptor complex under control conditions was estimated to be 90 min.

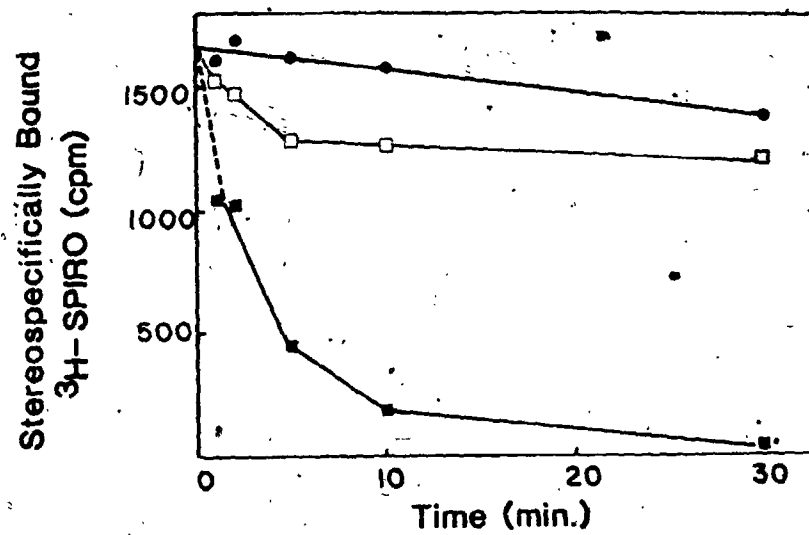


Figure 7.8

Morphological appearance of striatal membranes.

Striatal homogenates were preincubated for 1 hr., 37°C, in the presence of no metal (Control; Fig. 7.8a,b), 3mM Hg^{2+} (Fig. 7.8c,d), or 3mM Mn^{2+} (Fig. 7.8e,f) and later processed for electron microscopy. The control homogenates were characterized by the presence of abundant vesicular bodies many of which were judged to be synaptosomes (closed arrows). Membranes from Hg^{2+} treated homogenates were broken and fragmented. Vesicular structures, when present, were usually devoid of contents. Fig. 7.8d depicts what may be the remnants of a synaptic cleft (open arrow). Membranes from Mn^{2+} treated homogenates retained the general characteristics of the control membranes with the presence of numerous synaptosomal-like structures (arrows).

Magnifications: Fig. 7.8a (25,000X)

Fig. 7.8b (60,000X)



a



b

Figure 7.8c

Magnification: 25,000X

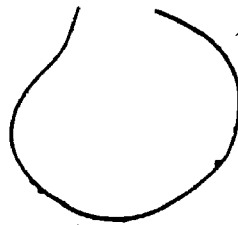
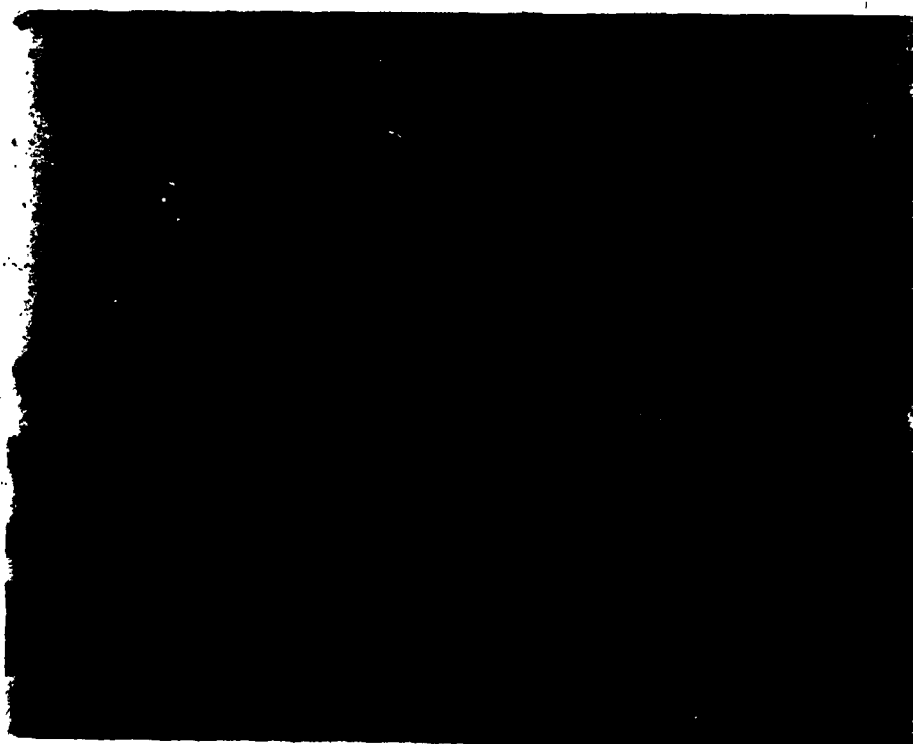


Figure 7.8d

Magnification: 50,000X



C



D

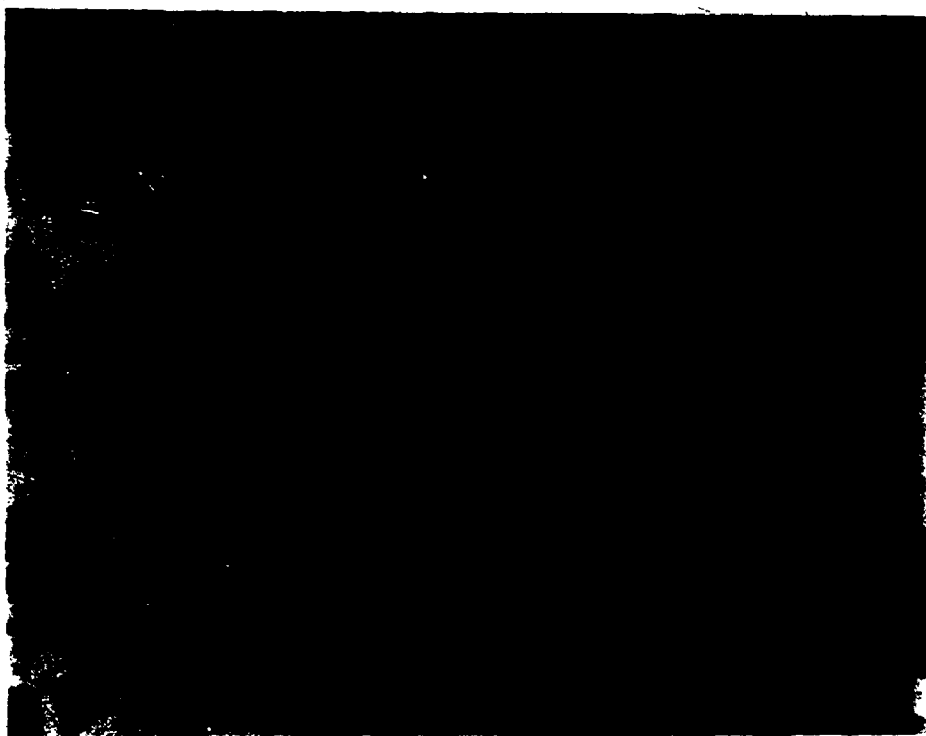
Figure 7.8e

Magnification: 25,000X

Figure 7.8f

Magnification: 60,000X

e



f



CONCLUDING REMARKS

In the preceding series of investigations, I have explored several aspects of the metabolism and kinetics of Mn in the rat. Although some questions regarding the binding, accumulation, elimination, and biological effects of Mn have been answered by means of these studies, basic information is still lacking in many areas involving the biology of Mn. These unanswered questions will, hopefully, form the nucleus of future research efforts aimed at reaching a clearer understanding of the normal metabolism and toxicology of Mn. The following is a list of subjects which I feel are among the most important in the ongoing search for information on the toxicology of Mn:

1. What factors (genetic makeup, nutritional status, age, etc.) are important in determining the individual susceptibility observed in manganese neurotoxication? Is there a portion of the population which is at especially high risk and, if so, can we develop simple procedures to predict this?
2. What are the effects of higher than normal Mn exposure in utero? In light of data which indicates an interaction between Mn and catecholamines, what is the effect of Mn exposure on developing catecholaminergic pathways in the fetal CNS?
3. What accounts for the psychiatric symptoms which often precede the neurological dysfunction associated with manganism in miners and why are these psychiatric manifestations not observed in cases of manganese poisoning in the U.S.A.?

4. Are there stages during the development of chronic Mn poisoning when the syndrome is reversible? Can specific chelating agents be developed which would facilitate recovery in patients suffering from this affliction?
5. Since, under occupational conditions, elevated exposure to Mn will probably be accompanied by elevated exposure to other substances - most importantly, other metals - do certain combinations of these substances present special health hazards?
6. Can behavioral tests be developed that will provide sensitive methods for the prediction of corresponding neurochemical alterations caused by chronic Mn exposure? Could such tests prove valuable in the early detection of Mn intoxication in humans?
7. What is the relative importance of direct absorption from the lung, as opposed to the GI tract, as a route of absorption of inhaled Mn?
8. What is the pattern of absorption, brain accumulation, neurochemical effects, and excretion of methylcyclopentadienyl manganese tricarbonyl and other organomanganese compounds used as fuel additives? Generally, organometals are accumulated by the CNS more readily than their non-organic forms due to increased lipid solubility (e.g. Pb, Hg).
9. What are the effects of chronic Mn exposure on GABAergic and cholinergic parameters in the CNS? What is the primary neurochemical lesion in Mn poisoning?

10. What are the molecular mechanisms responsible for the rapid clearance of Mn from plasma by the liver and the subsequent efficient excretion of Mn via the bile? What are the important intracellular Mn-binding ligands?

Hopefully, future research will provide the answers to these and other important questions regarding the biology of Mn.

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